

NIH's first strategic plan debated

Described by its leader as a \$9-billion dollar corporation, the US National Institutes of Health are giving long-range corporate planning for biomedical research a try.

THE US National Institutes of Health (NIH) took a first public step toward developing a 'strategic plan' for research funding when it revealed the draft of a year's effort last week at a meeting in San Antonio, Texas. At heart, the plan is being written to justify arguments for greatly increased federal funding for the NIH but, as NIH director Bernadine Healy expects, it will also bring into the open a number of fundamental (if contentious) questions about the structure of the NIH enterprise itself.

Implicit in serious strategic planning are questions such as these:

- Should NIH spend hundreds of millions of dollars on clinical trials that could be paid for by industry? Much of the NIH AIDS budget, for example, is spent on clinical studies, not basic science.

- Should the intramural research enterprise located on the NIH campus in Bethesda, Maryland, be continued? Many NIH facilities are in need of costly repair. And, whereas NIH was once the centrepiece of biomedical research, during the past 30 and more years, it has ably spawned new centres of research excellence throughout the country. Nonetheless, intramural NIH remains the one place scientists can work without either the burdens of grant-writing or the obligations of teaching, thus arguing in its favour.

- The draft plan proposes that one of the NIH's goals should be the expansion of research 'in order to enhance the Nation's economic competitiveness'. As they say in Texas, 'Them's fighting words,' to scientists who see their mission as the pursuit of knowledge, not money. If NIH's goal is to foster competitiveness, the resources it puts into technology transfer and patents are well spent. Alternatively, if that goal is rejected by the research community, the funding implications are also clear.

- Intellectual capital, otherwise known as young scientists, is also central to strategic planning. If there is an NIH mission to train the next generation, Healy says 'we're going backward,' with less money for training.

Strategic planning also raises, inevitably, the notion of consensus that some areas of research are so important, or intellectually ripe, that they deserve funding increases at a rate that is disproportionate to (that is, higher than) others. Healy is plainly sympathetic to this view, but the biomedical community as a whole—favouring the strategy of every discipline on its own—has never successfully reached a consensus on research priorities.

These are but some of the real issues that underlie strategic planning—issues that barely penetrated the surface in San Antonio but that explain nonetheless why the NIH's draft plan could be expected to generate a volatile response. Which indeed it has.

The unveiling of the draft, scheduled to coincide with the fiftieth birthday of the Southwest Foundation for Biomedical Research (known for the valuable primate work it does) brought to San Antonio some 60 hand-picked senior scientists from throughout the United States assembled to review the planning documents, written by the NIH leadership during the past year and now, for political reasons, referred to not as a plan at all but as an easy-to-like, reader-friendly 'framework for discussion'. The discussion had its ups and downs.

The scientists-cum-peer reviewers, perhaps genetically programmed to eschew anything that smacks of 'targeted' research, arrived in San Antonio in an apprehensive mood, exacerbated by the fact that NIH officials were so slow to get background documents out to meeting participants, (who were assigned to one of five panels), that few felt prepared for the task. Others, who had been denied access to papers for panels other than their own, arrived convinced that NIH's claim to want substantial comment to its draft plan was nothing but a public relations ploy.

This charge is one that Healy resolutely denies. 'The whole purpose of this is to get input from the research community,' she says. 'NIH cannot do this alone.' Two days in San Antonio made that evident.

Dissatisfaction with NIH's handling of the meeting spilled over to dissatisfaction with the background documents themselves. Cast under five headings—critical technologies, research capacity (which no one could define), intellectual capital, stewardship of public resources, and public trust—they failed to pass peer review.

However, as the debate wore on, some consensus became evident. It was generally agreed, much to some people's surprise, that the idea of strategic planning is a good one and should not be abandoned because it got off to a bad start. The mood was also decidedly supportive of Healy. The sentiment that it is important to the entire enterprise that Healy and NIH succeed was commonly expressed. And in essence this is right. At present, NIH is beleaguered enough by resources that are inadequate to research demand and congressional committees that question its integrity. It does not need assault from within.



However, it is evident that a strategic plan that even begins to deal with the tough questions cannot be written by researchers at regional meetings. Once the current exercise in participatory democracy is concluded, (four meetings open to the research community at large are scheduled for the next few weeks), Healy should bring the effort back to NIH, but not to closed meetings of institute directors. Rather, strategic planning should be handed over in its next phase to the institute councils, particularly the one that advises the director. If the hard questions can be framed and openly debated, strategic planning will have served a useful purpose. If researchers can, by laying out priorities, convince Congress to spend more on biomedical research, so much the better. But it will all be for naught unless the research community is willing to put its mind to the task of making choices. □

Swiss conundrums

Next Sunday's referendum on animals in research could damage Swiss research, academic and industrial.

SWITZERLAND, by European standards one of the most rational, cultivated and prosperous of states, appears to be on the brink of an uncharacteristic act of waywardness. In Sunday's referendum, the population will be asked to declare itself for or against the use of animals in research (see page 575). But not in as many words. Those who have devised the proposition have cleverly cloaked their intentions in a show of reasonableness: research using animal experiments will continue to be allowed if it can be shown to benefit directly the welfare of people or other animals. What, puzzled voters will be asking on Sunday, can be wrong with that?

Several things. If passed on Sunday and then literally translated by the federal government into legislation, Sunday's proposition will be the death of much academic research. What, for example, if researchers wishing to make antibodies against proteins involved in gene regulation confess their need to use mice or rabbits in the process? They may argue that a better understanding of gene regulation will eventually benefit the practice of medicine, but that benefit will hardly be direct. Alternatively, they may persuade collaborators or commercial laboratories outside Switzerland to make their antibodies in return for some appropriate consideration. But many researchers, faced with such a prospect, will prefer to work in some other field or to emigrate from even Switzerland's lush pastures. Is such an impoverishment of Switzerland's research enterprise what the people really intend?

One paradox is that Switzerland already enjoys some of the most careful legislation on the use of animals in research. And, given the noise that animal welfare groups have been making, the enforcement of the legislation has become noticeably tighter in the past few years, to the point at which research group leaders at commercial laboratories are now accustomed to telephone invigilation by animal welfare supervisors at Berne, the federal capital. To the extent that researchers are thereby vividly

reminded of their responsibilities, few can complain, but over-zealous regulation can be as serious an impediment to research as over-intrusive legislation.

Another, and more puzzling, paradox is that much of Switzerland's wealth derives from a handful of successful multinational pharmaceutical manufacturers, whose indigenous research can only be hampered if Sunday's proposition gives animal welfare groups the right to challenge in the courts experimental protocols involving animals. Although most research related to the development of drugs is relevant to human welfare, the pharmaceutical companies rightly fear the nuisance likely to be licensed by legislation if the proposition is accepted. For the rest of us, it is already remarkable that Swiss environmentalists, from their stronghold in the canton of Basle, have fallen into an antagonistic relationship with their most successful industry. To give them a licence to go further will be yet another proof that only the very rich can afford to indulge in such self-impoverishment. □

Patron of science

Jack Whitehead, wealthy from success in technology, gave his energy and resources to pure science.

EDWIN C. (Jack) Whitehead died on 2 February at the age of 72. He was playing squash. Whitehead, an energetic, feisty, and persistent man of great wealth, is best known as the founder of the Whitehead Institute at the Massachusetts Institute of Technology. Now a decade old and well established as a centre of intellectual power in molecular biology (and until recently headed by his friend David Baltimore), the institute is testament to Whitehead's belief that real advances in medicine and biotechnology derive from research into the structure and mechanisms of living things. Although his wife, Elizabeth Jones, to whom the library at the institute is dedicated, died of breast cancer, Whitehead was never lured by the idea that disease can be cured by targeted research.

But, his desire to create an institute for basic science took years to fulfil. More than one medical school declined Whitehead funds on grounds that there were too many strings attached, leading him to comment that "it is easier to make \$100-million than to give it away".

Whitehead, who made his considerable fortune from Technicon, a company he and his father started in 1939 that marketed the first blood autoanalysers to medical and research laboratories, did not put all his energies into the Whitehead Institute. Particularly after he sold Technicon to Revlon for \$400-million in May 1980, he channelled much of his attention to the US National Library of Medicine, where he headed a group of prominent citizens dedicated to the library's support, and to a fledgling organization called Research! America whose mission is to teach the US Congress and public alike that the treatment and cure of disease lies in basic research.

Whitehead should be remembered as one of this century's most important patrons of pure science, of whom there are entirely too few. □

Swiss vote Sunday could restrict animal research

- Referendum backed by activist groups
- Relevance to health must be shown

London

Swiss biomedical researchers fear that their work with animals could be sharply curtailed if voters next Sunday (16 February) approve a referendum on animal experimentation. The Swiss Animal Protection League is asking voters to support an initiative that would allow animal research only if it can be shown to be directly relevant to human or animal health. Researchers are worried that, if the referendum passes, animal rights groups could disrupt most research with animals by making use of a new right to challenge research projects in court.

Under Switzerland's somewhat obsessive democracy, any proposition backed by more than 100,000 signatures can be put forward for a public vote. The federal government is obliged to give legislative form to propositions carried at public referenda.

Sunday's referendum is not the first on animal experimentation. A proposal for a total ban, put forward in 1985 by Franz Weber, a prominent figure in the Swiss wildlife conservation movement, was thrown out by a large majority. But Sunday's proposition is worded much more subtly. Researchers fear that many Swiss will believe the initiative offers sensible controls on the conduct of experiments and fail to understand its full consequences.

Paul Walter, a University of Basel biochemist and president of the pro-research

group *Arbeitskreis für Gesundheit und Forschung*, says that passage of the referendum places basic research "very much in danger". Even more serious — at least in terms of the Swiss economy — is the effect that the initiative would have on the country's pharmaceutical industry. Switzerland is home for several world-leading companies, including Hoffman-La Roche, Ciba-Geigy and Sandoz. Industry research laboratories account for about 80 per cent of the animal experiments now approved in Switzerland each year.

Heinz Weber, animal welfare officer at Sandoz in Basel, says that his company's research would be seriously disrupted if animal welfare groups could challenge in court the ethics of individual experiments. Drug development is a sequential process, he says, and if certain key experiments are delayed, "then the whole process is stopped". Walter describes the proposed right of legal challenge as "murder for experimentation", saying that work could be delayed for as long as two years while courts deliberate. He fears that extremist groups, as a matter of principle, will challenge virtually every experiment.

Supporters of the Animal Protection League's initiative reject Walter's assessment. Christoph Reinhardt, a researcher seeking alternatives to animal experiments at a private institute based in the Swiss Federal Institute of Technology (ETH) at Zurich, says the Swiss federal govern-

ment would nominate the organizations to be given the right to challenge planned experiments — and exclude groups likely to abuse the right.

But Alfred Schweizer, animal welfare officer with Ciba-Geigy in Basel, says that there are few moderate animal welfare groups in Switzerland. "If only 10 get the right, then 7 will be extreme. We have to prepare for the worst case."

If the initiative is passed, pharmaceutical companies are expected to begin moving research teams out of Switzerland. Weber says that Sandoz would consider moving projects to its laboratories in Britain, Japan and the United States. Apart from the direct restrictions on research, pharmaceutical companies are also worried by what the initiative implies for commercial confidentiality. If animal welfare groups are allowed to challenge individual research proposals, companies will presumably be forced to release details of their research plans.

Researchers also point out that the initiative contains a sting in the tail: if within five years the Swiss government fails to enact its provisions in a new animal experimentation law, animal research would be banned completely. A spokesman for the Animal Protection League describes the clause as a "time bomb" designed "to tell legislators that they'd better do something about it". But Walter says that enacting new legislation in Switzerland can be ponderously slow; he doubts the legislature's ability to meet the deadline, and says the Animal Protection League's initiative could bring "abolition by a very big back door".

Both sides expect Sunday's vote to be close, and have launched intensive media campaigns. The Animal Protection League has held some 15 press conferences. In response, Ciba-Geigy has decided on an 'open house' policy, inviting journalists and members of the public to tour its Basel laboratories. Schweizer, the company's chief animal welfare expert, argues that the initiative would actually hinder animal welfare. Experiments would still take place, he says, but in countries where animal experimentation laws are more lax than in Switzerland.

Nevertheless, some opinion polls show a slight majority in favour of the initiative. Walter expects support to vary markedly between the 25 cantons that make up the Swiss federal republic. He concedes that cantons with large urban populations are likely to support the initiative, but hopes that the sparsely populated agricultural cantons will come to the aid of the animal research community. Swiss farmers have little sympathy with animal welfare activists, he suggests, and the Animal Protection League has to win support for its initiative from a majority of the cantons, not just a majority of the electorate.

Peter Aldhous

Video lessons for UK drug researchers

London

A NOVEL interactive video system to train new researchers and technicians in animal care and surgical techniques — before letting them loose on live animals — has been launched by the Association of the British Pharmaceutical Industry (ABPI). The video system should be a standard component of British drug companies' staff training within 12 months, says David Clough, research director at Roche Products and chairman of the ABPI's animal welfare committee.

The system so far comprises six 30-cm video laser disks, each dealing with specific topics. It can run on a standard personal computer, linked to a video disk player. ABPI claims that its system is the most comprehensive package of its type, and the first to use the relatively

new technology of interactive video.

The system goes a long way towards meeting the requirements of the 1986 Animals (Scientific Procedures) Act, which made training a central theme. The act also introduced a system of licences to sanction work on specific projects, and by specific researchers. By autumn 1993, the Home Office inspectorate responsible for policing the legislation wants researchers to have completed a training course before they apply for a licence. British drug companies are expected to begin using the system soon. But the cost — £4,000 for the computer/video disk player work station and another £2,500 (plus Value Added Tax) for the six disks themselves — is expected to pose more of an obstacle for academic laboratories. **Peter Aldhous**

Will aplenty, but is there a way?

Chicago

As the science machinery of the former Soviet Union continues to crumble, there are no shortage of ambitious schemes to save it: last week alone, the chairman of the US House of Representatives science committee unveiled a proposal to set up a \$200-million foundation to sponsor peer-reviewed research in Russia, and Administration officials revealed that they were hoping to set up several centres to find and support nondefence research for scientists now working in the country's military research centres. Among the other initiatives is a congressional bill that would spend a portion of \$400 million saved from US defence cuts to bail out Russian science, and discussions involving several private foundations as to how they can best do their part.

In an initiative unveiled last week at the annual meeting of the American Association for the Advancement of Science (AAAS) in Chicago, Representative George Brown (Democrat, California), chairman of the House Science, Space and Technology Committee, announced plans to introduce legislation that would create a \$200 million endowment to fund peer-reviewed research in Russia. Called the "US/Russian Science & Technology Foundation", Brown's proposed fund would require matching contributions (in rubles or indirect support) from the two govern-

ments, much like the joint research foundations that now exist between the United States and Israel. Brown also proposed a similar foundation to support industrial research and development, providing partial funding for high technology ventures linking Russian entrepreneurs with US business partners. The science committee staff plans hearings on both initiatives later this year.

But when it comes to real money, here and now, Russian scientists appear to be far down the priority list, after food, humanitarian aid, arms control measures and economic training. Any substantial US support of basic research in the former Soviet Union appears to be a year or more away.

Even among the sectors that do have Russian science on their list, little seems likely to happen soon. Part of the problem is simply that such large efforts take time; even if the congressional bills are eventually approved, money is not likely to be available until next year. There is also some uneasiness about sending large sums to support Russian researchers when US researchers in many fields are struggling.

But perhaps the most difficult problem to resolve is how, exactly, to ensure that money for Russian science goes to the right people. Research in the Soviet Union has traditionally been funded in classic Soviet fashion, with a central policy-mak-

ing body dividing up the science budget into block grants to various institutions. Directors of those institutions generally allocated those funds as they saw fit, which often meant that the best-connected and most politically aware scientists got the most money. Despite the political changes in the country, this is still relatively unchanged.

Many of the new proposals to aid Russian science aim to circumvent these remnants of Soviet bureaucracy. Brown's proposal, like many others now being considered, would sidestep the 'top-down' funding approach, in favour of individual grants to scientists on the merits of their research alone. He foresees a peer-review panel of Western scientists who would decide which joint US-Russian proposals to fund. This is an idea endorsed by many Russian researchers, who see outside appraisal as the only way to avoid the traditional morass of favouritism and connections. "It is most important to determine the quality of the science," Evgeny Sverdlov, director of the Moscow-based Institute for Molecular Genetics, told a panel at the AAAS meeting. "Otherwise the true scientists will be killed off and the idlers will remain because they have relatives in government."

Peer review is not an issue for Secretary of State James Baker, who is expected to offer a plan to help Russian defence

Stopping the Russian nuclear brain drain

ABOUT 2,000 military scientists of the former Soviet Union are expected to be offered employment in an international scheme for the safe decommissioning of surplus nuclear weapons. The scheme is intended to discourage the scientists from accepting jobs with the armaments programmes of aggressive developing countries. An agreement was reached in principle two weeks ago at a meeting between Presidents Bush and Yeltsin in Washington.

Professor Valeri Mikhailov, publicly identified for the first time two weeks ago as the director of Russia's nuclear weapons establishment, said in Moscow that up to 15,000 specialists employed in the programme had access to classified information on plutonium production and uranium enrichment techniques and about 2,000–3,000 knew a great deal about nuclear bomb design.

The elite military scientists had until recently enjoyed considerable privileges. They now earn salaries worth less than \$150 a year at the official rate of exchange. Former Soviet weapons scientists have been offered up to \$400,000 a year by developing countries, payable through secret Swiss bank accounts, according to Augusto Forti, director-general of the Venice-based European Institute for East-West Cooperation.

Although they are still subject to a five-year ban on emigration, many observers doubt whether it is enforceable in the present state of transition. Mr Roland Dumas, the French foreign minister, has now returned from a visit to the

four nuclear republics where they are employed and where he tried to persuade the authorities to enforce that ban vigorously.

The scientists are expected to be offered work by a projected new international scientific research centre. The plan coincides with warnings of the danger of radiation leaks — which Mikhailov says could turn the country into "one big Chernobyl" — arising from an inadequately executed programme for the dismantling of unwanted nuclear weapons. Britain and the United States have already promised specialist military assistance and technological support for the decommissioning programme.

The employment plan was formulated largely by Germany, Britain and the US. Mr Hans-Dietrich Genscher, the German foreign minister, has warned that the unemployed military experts are "wandering technological mercenaries" who could fall "easy prey to recruiters for irresponsible potentates with ambition to build weapons of mass destruction". Mr Richard Cheney, the American Defence Secretary, has named Iraq and North Korea as countries likely to compete for Soviet-trained scientists; Western analysts also worry about Syria, Libya and Algeria as well as Iran. Britain raised the issue at the recent Washington conference on aid to the former Soviet Union.

Foreign headhunters have already staged some well publicized operations in the former Soviet republics of Central Asia near Iran, where several military research and development complexes are based. Morale at the weapons establishments is low, with salary cuts of 40 per cent being imposed and a staff cut of up to 60 per cent expected.

Thomas Land

scientists when he meets Russian President Boris Yeltsin this week. Although the details of the plan are sketchy, it is expected to provide for Western-financed 'clearinghouses', in which researchers in Russia's crumbling defence industry could be matched with Western companies, research groups and government agencies that could use their talents. This approach is meant to encourage scientists to remain in Russia, and not share their weapons knowledge with developing nations.

But this proposal would apply to only a few thousand of the estimated one million scientists in the Russian research establishment. And some US policy experts are concerned that it might send the wrong message to that community. "Those researchers who made a conscious and moral choice not to work in the Soviet defence industry should not be ignored," says Harley Balzer, director of the Russian studies programme at Georgetown University in Washington, DC.

Other projects intended to help the former Soviet Union appear to ignore Russian science altogether. A bill in Congress that would give the US Agency for International Development (AID) \$645 million to provide the former Soviet Union with humanitarian and "technical" aid would not include research funds, according to Hiram Larew, a member of the AID strategic planning office. "There has been virtually no mention of research activities in the current [AID] plan" to give aid to the region, he told the AAAS panel.

One of the few concrete plans for Russian science is not coincidentally one of the smallest. The AAAS international office has decided to focus on helping Russian institutions to resume their subscriptions to foreign scientific journals. In January the Russian Academy of Sciences cut the funds for all its institutes' subscriptions, which virtually isolates most Russian researchers from developments in the West. Although several journals (including this one), have agreed to continue Russian subscriptions at cost, money for even that reduced expense is not available in Russia, and has so far been slow in coming from the West. AAAS plans to use a \$2,000 grant from the Chicago-based MacArthur Foundation to survey scientific societies and leading journals to discover how many subscriptions have been affected by the Russian measure. Association officials hope to raise several million dollars from private foundations to refill Russian bookshelves.

Despite the limitations of current proposals, there is no shortage of goodwill towards Russian science. Individual efforts from US researchers have gone as far as food shipments and personal cheques. But short of direct contact with people in Russia, there are few mechanisms to ensure that private donations will get into the right hands. **Christopher Anderson**

Tragedy revealed in Zurich

London

A TRAGIC case of scientific misconduct, revealed last week by the University of Zurich, underlines the importance of ensuring that key data are replicated reliably before publication and that several members of a research team are able to carry out difficult experimental procedures.

The case centres around a claim, first published in the *EMBO Journal* (9, 385; 1990), that researchers at the University of Zurich's Institute of Molecular Biology had produced functioning measles viruses by microinjecting cultured human cells with cloned complementary DNA (cDNA) produced from the RNA genome of the virus. The data underlying the claim, provided by Isidro Ballart, a South American PhD student working at the institute, were later found to be fraudulent. On 20 March 1991, shortly before evidence of the fraud began to emerge, the student was found dead in his laboratory.

Martin Billeter, the leader of the research group, last autumn retracted the *EMBO Journal* paper as well as a second publication, based on the fraudulent data, that appeared in the *Journal of Virology* (65, 3161; 1991). But the university was able to reveal the case only last Friday (7 February), having been sworn to secrecy until a Zurich district attorney had completed a full inquiry into Ballart's death. His death has been ruled a suicide.

The *EMBO Journal* paper caused a stir among virologists. The study of negative-strand RNA viruses (in which the single strand of RNA must produce a complementary copy of itself inside the host cell before viral proteins can be transcribed) has been hindered by the lack of an experimental system for genetic manipulation. Using the model system Ballart developed, Billeter's group aimed to produce genetically engineered measles viruses by manipulating the cDNA injected into the cultured cells. Their aim was to study the function of individual measles genes, in particular the mutant genes associated with a fatal disease of the central nervous system, subacute sclerosing panencephalitis, which occurs in about one in 100,000 cases of measles.

The model system might also have allowed the production of new genetically engineered vaccines. "It would have been the most important breakthrough in our area of virology for the past 10 years," says Dan Kolakofsky, from the University of Geneva.

But the euphoria in Billeter's group came to an abrupt end with the discovery of Ballart's body. At first, it was thought he had died from natural causes. A routine examination by the university's Institute of Forensic Medicine found no evidence of poisoning. But a subsequent detailed

autopsy revealed that Ballart had taken his own life by swallowing a quantity of "a common inorganic chemical", says Charles Weissmann, director of the Institute of Molecular Biology.

Meanwhile, other PhD students in Billeter's group were discovering that viruses purportedly produced in cell culture from cloned cDNA were no different from the standard Edmonston measles virus strain used in the laboratory. At first, nobody suspected that the dead student was guilty of fraud, instead assuming that the cell cultures must have been inadvertently contaminated with the laboratory strain. "He was very popular," says Billeter, and "always willing to help his colleagues."

But when other members of the group consistently failed to replicate Ballart's results, Billeter and his colleagues began to work systematically through the preparations left behind. The evidence pointed to one conclusion: the student had not been able to produce viable viruses by microinjecting cultured cells with cloned cDNA; instead, he had simply infected the cell cultures with the standard lab-strain virus. His genetic analyses, showing marker genes present in the cDNA, came not from viral cell culture, but from the original cDNA samples. Weissmann says that the fraud went undiscovered until other members of Billeter's group began conducting their own genetic analyses, using viruses supplied by Ballart.

In July 1991, Billeter wrote to his colleagues in the field, explaining the circumstances of the fraud; published retractions soon followed. In August, Weissmann and Billeter also pressed the university's rector to appoint an external commission to sift through the data. But the university took no further action after Clive Kuenzle, a biochemist and one of the university's pro-rectors, concluded that the evidence indicated clearly that the dead student had acted alone.

Virologists agree that Billeter was diligent in informing his colleagues in the field about the fraud. Still, the effect on Billeter's group has been debilitating: the researchers involved went through "a year of catharsis", says Kolakofsky.

The tragedy also points out the problems that can arise when experiments hinge on the performance of a difficult technique by a single relatively inexperienced researcher. For more than a year, Ballart alone was responsible for microinjecting cDNA into cultured cells and for performing the genetic analyses supposed to show that the viruses produced in the culture were derived from the cDNA. "I'm sorry that I didn't insist at the time that other members of my group also learn to do the microinjection," says Billeter.

Peter Aldhous

US research could lose lustre

Washington

THE Bush Administration is planning to downgrade its four-year-old Global Climate Change research initiative at a time when there is new concern about ozone depletion in the Northern Hemisphere. The shift in focus away from global change, announced last week by presidential science adviser D. Allan Bromley, will make it harder for the \$1,100-million research effort to continue its rapid growth of the past few years.

Bromley told members of the President's Council of Advisors on Science and Technology at their monthly meeting that global change research has "matured" to the point where it should place greater emphasis on assessing the results of the research already carried out. Much of that evaluation would be done by groups outside the government, he said, such as the National Academy of Sciences.

The 1993 budget proposal submitted last month to Congress contains five presidential research initiatives—science and mathematics education, biotechnology, high performance computing and advanced materials processing, in addition to the global change effort. They represent the combined research budgets of several government agencies in a particular field, knitted together by an overall research strategy.

The initiatives on biotechnology and advanced materials are new this year. Bromley said the work involved in preparing integrated budgets for each of these initiatives makes it difficult for the government to support more than five at any one time. He said that global change is the logical one to drop because it is the oldest. The global change programme would be moved to the new category of a national

research programme, according to Bromley. He said its new status "in no way is a de-emphasis" of the topic.

Robert Corell, assistant NSF director for the geological sciences and co-chair of the committee that oversees the global change effort, does not think that any change in status will hurt the programme. He said that the recent discovery in the Northern Hemisphere of atmospheric conditions that are ripe for rapid ozone depletion demonstrates that the programme is producing results that can shape federal environmental policies. "I can't imagine any agency deciding to do less than it is doing already just because global change isn't a presidential initiative," he said.

Without its status as a presidential initiative, Bromley pointed out, the global change programme would no longer qualify for "fencing" in future budget submissions. That would make it easier for the directors of the various agencies now participating in the programme, notably NASA and the National Science Foundation, to move money into different research projects. "If it's not a presidential initiative", Bromley said, "the probability of continued major increases in funding will be lower."

Bromley said the issue would be raised at a meeting on 2 March of the Federal Coordinating Committee for Science and Technology, the interagency panel that oversees all federal research. He said that the committee would review proposals for a new research initiative for the budget of the fiscal year 1994, to be submitted next January, and acknowledged that a proposal on advanced manufacturing had a great deal of support among federal science administrators.

Jeffrey Mervis

Back from the dead

London

As if a phoenix from the ashes, palaeontologist V. J. Gupta has been reinstated as a member of the faculty of the Panjab University of Chandigarh, in northern India. Other members of the university expect that it is only a matter of time before he is also reinstated as head of his department. Gupta was suspended from both positions in February 1991 after repeated allegations that many of his published research papers described fossils that had not come from the sites in the Himalayas where he said he had found them, but often from other people's collections and even fossil shops in Paris.

Gupta's chief critic has been Dr John A. Talent of MacQuarie University in New South Wales (see *Nature* 338, 613–615; 1989), but Talent's conclusions have been corroborated by other palaeontologists outside India.

Although Gupta has consistently denied improper use of evidence, a different opinion appeared in the *Journal of the Geological Society of India* in January last year, based in part on a field expedition to the disputed fossil sites. Since then there have been two other India-based investigations, by the National Science Academy of India and by a field expedition last September, led by Dr A. S. Paintal.

Gupta's reinstatement last week was determined by the university's governing body, a subcommittee of the Senate known as the Syndicate, and by the vice-chancellor of the university, Professor T. N. Kapoor. The Syndicate's decision appears to have been influenced by a 1,000-page defence against criticisms produced by Gupta as well as by the opinion of a retired judge of the High Court of India, neither of which has been made public.

John Maddox

US NATIONAL LABORATORIES

Beating swords into "critical technologies"

Chicago

LAWRENCE Livermore National Laboratory, birthplace of "Star Wars" and a host of advanced nuclear weapons, would be turned into a billion-dollar technology development laboratory under a new plan announced last week by Representative George Brown (Democrat, California), the chairman of the House science committee. The idea, which is certain to be opposed by the Bush administration, is best seen as part of a campaign by technology supporters to foster debate within Congress about a new role for the multi-billion dollar labs.

In a 8 February letter to James Watkins, secretary of the US Department of Energy (DOE), Brown argues that the United

States, in the aftermath of the Cold War, needs two technology development laboratories more than it needs three nuclear weapons laboratories. Under his proposal, all nuclear weapons work would be transferred to the Los Alamos National Laboratory, with Livermore converted into a "National Critical Technologies Laboratory". The shift, Brown says, would permit Livermore scientists to work more closely with industry and academic institutions in such areas as materials science, environmental remediation, and fusion and biotechnology. Sandia National Laboratory, the third of DOE's main weapons laboratories, would build upon its strengths in measurement and engineering by transferring civilian technologies

to industry, as well as developing arms control verification and monitoring systems.

DOE's nuclear weapons budget, now almost \$2,000 million a year, would be cut 20 per cent each year for the next four years, under Brown's plan, until it reached an annual level of \$750 million by 1996. The money saved could be spent on civilian technologies.

All three labs, seeing the post-Cold War writing on the wall, have been scrambling to diversify from weapons work to civilian and environmental technology. Livermore, for example, has shifted about half of its programmes to non-defence research.

Christopher Anderson

Battlefield in Flanders over funding

London

In a display more reminiscent of militant French farmers than sober Belgian researchers, some 5,000 Flemish scientists took to the streets of Brussels last Thursday (6 February) to protest against the science policy of the regional Flemish government.

The academic pressure group that organized the demonstration, Focus Research, says that Belgian academics have always been poorly funded compared with their neighbours in France, Germany and the Netherlands. But the group claims that the situation for Flemish academics has gone from bad to worse since 1988, when Belgium moved sharply towards a federal constitution that gave the regional governments in Flemish-speaking Flanders and French-speaking Wallonia effective control over the country's science policy.

The two communities come from different cultural traditions. The Flemish north identifies closely with the Netherlands, while the Walloon south is tied to France. By 1988 the regions' economies had diverged strongly, with Flanders emerging as a centre for high-technology ventures, while Wallonia struggled on with its traditional heavy industry.

The solution then was to divide Belgium into three semi-autonomous regions: Flanders, Wallonia and bilingual Brussels, the state capital and administrative centre of the European Communities (EC). Since then, responsibility for domestic policy — including science — has been passed in large part to the regional governments. In Flanders, for example, the Flemish government now controls about 80 per cent of public funding for science in the region.

Kris Thielemans, an immunologist from the Flemish Free University of Brussels and president of Focus Research, believes this federalization has damaged Flemish science. Since 1988, he says, the Flemish government has neglected fundamental research in the universities at the expense of several costly and high-profile applied research programmes. (Following the lead of the EC, schemes to underpin the information technology industry have been prominent.)

Last week's unprecedented show of public dissent was triggered by two events late last year. In December, the Flemish

government decided to slash its funding for high-energy and nuclear physics. At the same time, it ended a scheme that offered tenured research-only positions at universities for the cream of Flemish scientists. The 200 people already benefiting from the scheme will not be removed, but they are expected to come under pressure to take up new jobs in industry, or as university lecturers.

Even more worrying, says Thielemans, is the negative message that the new policies have sent to recent graduates at a time "we need to attract more people". The cuts

ent advisory Council on Science Policy, sympathizes with Focus Research's protest. "Fundamental research has lagged behind. That's clear," he says. Roos argues that the Flemish government has also tried to meddle in the detailed management of Flemish research, putting political appointees into lowly administrative positions. Roos also points out that there is so far no equivalent to his Council in Wallonia.

But Roos believes that the sorry state of Flemish science predates the changes of 1988. "Federalization is not the cause of the problem," he says. Although many Flemish academics look enviously towards their counterparts in Wallonia, Roos says, the underlying situation is little different: in both regions, public spending on science as a proportion of national wealth is about half that spent by its neighbour, the Netherlands. Walloon researchers also are ripe for a protest march, he says, if their government made a single unpopular science policy decision.

The organizers of last week's demonstration (which was accompanied by strike action at Flanders' universities) believe that media coverage will help their cause. "People are talking about science," says Patrick Callaerts, a *Drosophila* embryologist from the Catholic University of Leuven. "That wasn't the case before." Indeed, the main criticism levelled in the Belgian press was that the demonstrators did not shout loudly enough. "It was very civilized," says Filip Volckaert, a zoologist colleague of Callaerts', who says that the presence of many senior university officials introduced a strong measure of restraint.

Flemish researchers are now waiting to see if their protest brings about change. A new coalition Flemish government was installed only in late January, after elections last November. (Attempts to form a coalition national government, from a parliament elected on the same polling day, are still foundering.) Eventually Focus Research wants the Flanders government to double its spending on research. One positive sign is that the new Flemish prime minister, Luc van den Brande, has promised to take responsibility for the region's science policy. But this gesture must be backed by positive action to pacify Focus Research. "We'll be watching him," Callaerts promises. **Peter Aldhous**



Flemish scientists take to the streets of Brussels. Will the new regional government deliver for long suffering academics?

in nuclear and particle physics, while specific to those disciplines, are severe. Raymond Gaftmans, a theoretical high-energy physicist from the Catholic University of Leuven, says that some of his experimental colleagues will find their funds cut by as much as 90 per cent.

Thielemans is also concerned that the continuing process of federalization could make obsolete Belgium's main grant-making agency, the National Fund for Scientific Research. The fund's budget is already split in two — 55 per cent comes from the Flemish government and is spent in Flanders, while the remainder comes from the Walloon government, to support research in Wallonia. Given this division, replacing the National Fund with separate regional agencies seems logical enough, but Thielemans fears that the regional bodies might not follow the National Fund's system of grant evaluation. "We're afraid that a working system will be replaced by something new, with no experience of peer review", he says.

Geff Roos, a materials engineer from the Catholic University of Leuven who heads the Flemish government's independ-

Nuclear wastes seek home

London

WHAT is to happen to nuclear waste in Eastern and Central Europe? Nuclear plant operators there must decide very soon what to do with accumulating wastes now that the civil atomic power authorities of the former Soviet Union can no longer handle them.

Nuclear wastes generated by the Soviet-designed reactors used to be taken back to the Soviet Union, free of charge, for reprocessing. But a few years ago the Soviet government began to charge for this service. Late last year, the authorities announced that they would no longer accept any shipments.

Mr Hans Blix, director-general of the UN International Atomic Energy Agency (IAEA) in Vienna, has offered to mobilize help for the republics joining the post-Soviet Commonwealth of Independent States. At the same time, Sweden is helping Lithuania to set up an independent nuclear power authority, British Nuclear Fuels and France's Cogema are negotiating with East European governments while contractors from Western Europe, the United States and Canada are seeking business in the region.

The arrangements made for handling spent fuel may determine the fate of the 62 largely obsolete Soviet-designed nuclear

power plants, 17 of which are in the new Eastern and Central European democracies. A recent IAEA study described some of them as being in a dangerous condition.

A quarter of the electricity consumed in the territories west of the Urals is nuclear. The IAEA is concerned that, in the absence of a central regulatory authority, individual plant operators will be asked to shoulder too much responsibility for safety standards. Given the increased demand for nuclear electricity caused by acute shortages of other fuels and the already heavy economic and political stresses on the system, IAEA officials fear that such an approach is an invitation for disaster.

That there is chaos in the ex-Soviet nuclear power industry has been evident since the German Environment Minister, Klaus Toepfer, was told in Moscow late in October that the country simply lacked the means to take back spent fuel, contractual obligations notwithstanding.

Germany, Czechoslovakia and Bulgaria, saddled with the most dangerous Soviet-built reactors outside the commonwealth, as well as Hungary, must therefore find other solutions. Toepfer wants a joint Western approach to this huge problem. But a piecemeal approach seems more likely. Czechoslovakia, Bulgaria and Hungary are expected separately to announce

plans to build new dry storage facilities to house spent fuel for up to 50 years.

Germany has closed down the notorious Soviet-built Greifswald nuclear plant, consigning its spare parts to the Kozloduy plant in northern Bulgaria. That plant was also condemned by the IAEA study because of its shoddy safety equipment and frequent radiation leaks. The makeshift waste-storage facilities near the Bulgarian plant on the Danube are also dangerous and, in any case, are likely to be full in a matter of months.

Czechoslovakia, whose Bohunice plant was also condemned by the IAEA study as well as by safety investigators from neighbouring Austria, is also storing spent nuclear fuel near the reactors in unsafe conditions, according to an unpublished report commissioned by Greenpeace, the environment pressure group. The concrete used in the construction is said not to be waterproof, while the storage tanks were not designed for long-term use.

In Hungary, the technology, infrastructure and safety culture of its Paks nuclear power complex are highly regarded by IAEA. But the country's temporary waste storage facilities were judged to be inadequate. Some observers expect Hungary to embark on nuclear power generation for export to neighbouring Austria and Italy, countries that have set their faces against operating nuclear plants.

Thomas Land

BLIGHTED WOODLANDS

Environment project in Poland

THE first project to be financed from the \$1,400 million Global Environment Facility (GEF) is aimed at rescuing and upgrading vast forest lands in Eastern Europe affected by environmental pollution, including the infamous Black Triangle where Poland, Czechoslovakia and the eastern part of Germany meet. GEF is a new programme managed by the World Bank and financed largely by the industrialized countries of Western Europe, the United States and Japan.

Established at a Paris conference in November 1990, the fund is a three-year pilot programme providing grants and low-interest loans to poor countries (with a per capita gross national product beneath \$4,000 a year) to enable them to relieve pressures on global ecosystems.

The initial \$4.5 million grant financed by GEF will support Poland's efforts to protect its endangered forest ecosystems. The project includes plans for preserving woodlands, flora and fauna in the Sudety Mountains in the south-western part of the country as well as the Bialowieza Primeval Forest in the east.

The Sudety Mountains are in the centre of the ecological disaster area known as

the Black Triangle, where 13,000 hectares of forests in the Karkonosze region are dead and another 8,000 dying because of air pollution. The damage area is expanding eastwards.

About 58,000 hectares of the ecologically fragile Bialowieza forest are in Poland and the remaining 87,000 hectares in Byelorussia. This is Europe's largest expanse of natural forest, home of the European bison, lynx, wolf and the black stork.

The GEF programme in Poland and a tentatively agreed \$1m companion project proposed for Byelorussia will partly finance the construction of a gene bank, a greenhouse, seed orchards and equipment for collecting plants and seeds and monitoring air and soil quality as well as land use patterns. The grant will also provide specialist training and promote sound management and sustainable forest development policies.

The GEF programme is based on the recognition by industrialized countries that they must assist the developing countries in their efforts to sustain fragile ecosystems. Countries applying for GEF funds must be party to the Montreal protocol which restricts the production of substances

which deplete the ozone layer.

The fund has selected four areas for principal support:

- Biodiversity: The richest remaining sources of ecological systems and diversity of species are in the developing countries;
- The ozone layer: Developing countries will be assisted in making the transition from the use and production of chlorofluorocarbons and other gases which contribute to ozone depletion;
- Greenhouse-gas emissions: The adoption of cleaner fuels and technologies in the energy, agriculture and industry sectors.
- International waters: Investment for contingency programmes to prevent marine oil spills. GEF will contribute towards improved reception facilities for the removal of ballast water from oil tankers in ports and finance programmes to prevent or clean up the pollution of major waterways.

"If poor countries are to play their part in preventing further climate change and saving species, they will need special practical help", says Lynda Chalker, the British Minister of Overseas Development. "GEF should be strengthened and reinforced to provide the third world with the means to tackle global environmental problems."

Thomas Land

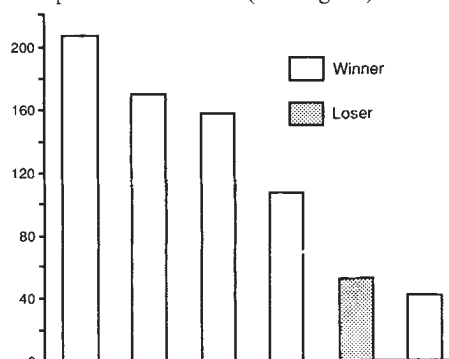
Academic promotion in Italy

SIR — As the actual winners pilloried in the letter on "Academic promotion in Italy" (*Nature* 353, 10; 1991), we should like to reply to the comment and assessments made therein by the author, who was one of the losers in the competition in question. His graph showed that his four selected losers had bibliometric evaluations more weighty than ours. Because of the well-recognized 'impact factor' of *Nature*, this article has been reported by several leading Italian newspapers with vignettes of a typical professor endowed with donkey's ears.

Bibliometrics may be widely used in the evaluation of research performance but, like statistics, it is nonetheless best left to experts. Thus a bibliometric analysis carried out over a long period of time will chiefly reflect past performance, instead of present scientific activity. Moreover, for academic promotion in a specific area, the appropriate number of candidate's citations must be that in this particular area, and not the whole field of medical science. These simple criteria were not respected in the analysis presented by the loser: a period of 23 years was evaluated, with no attempt to confine the citations to a specific field. Such an approach could, for example, enable an ophthalmologist on the verge of retirement to win a chair of urology.

We, nonexperts like the loser, have undertaken our own examination of the

bibliographic material. We carried out a very simple count of the number of citations provided by the *Science Citation Index* during the past seven years (1984–90) for the five winners and the loser in question. Interestingly, four of the winners had at least twice as many citations as the loser, whereas the fifth had a comparable number (see figure). It is



curious that these results are diametrically opposite to those found by the loser. This is just further proof that bibliometrics is a complex matter. This is not to deny the need for improvements in the complex machinery for academic promotion in Italy. But the thinly veiled complaints of a loser cannot but cast aspersions on the reputation of all holders of academic posts.

SERGIO AMADORI
CARLO BERNASCONI
MARIO BOCCADORO
ROSARIO GIUSTOLISI
MARCO GOBBI

Dipartimento di Medicina
ed Oncologia Sperimentale,
Universita di Torino,
Via Genova 3, 10126 Torino, Italy

Is anybody there?

SIR — In his favourable review of my book *The Cosmic Water Hole* (*Nature* 354, 334; 1991), Frank J. Tipler misrepresents my position on the issue of extraterrestrial intelligence. He states that "he [the author] admits that the search will probably fail because such intelligence probably does not exist". This does not correspond to anything I said in the book. I seriously consider the possibility that we are alone, but nowhere do I admit that the search will probably fail, and/or that extraterrestrial intelligence probably does not exist. In fact, the book was an attempt to convince people that these searches can benefit mankind, regardless of their outcome. Because the searches rest on an untested assumption, they have been (and still are) difficult to fund. While I don't contest Tipler's right to criticize the SETI projects, I do not want to share the responsibility of condemning and thus compromising them.

EMMANUEL DAVOUST

Observatoire Midi-Pyrénées,
14 Avenue Edouard-Belin,
31400 Toulouse, France

Sequence data

SIR — I read with interest your leading article "Free trade in human sequence data" (*Nature* 354, 171; 1991).

There are two main reasons why a biotechnology or pharmaceutical company might be interested in obtaining cDNAs and their sequences and there are several ways of getting them quite independent of any large-scale human genome sequencing initiative.

It is important to distinguish between cDNA sequences that code for proteins with potential therapeutic use and those that code for proteins, modulation of the activity of which might be used therapeutically. A useful paradigm for the former is the colony stimulating factors and of the latter the seven transmembrane receptor proteins and the ion channels. The cloning and sequencing of the cDNAs for many of these proteins have appeared in *Nature*. Generally, this

has been done either by cloning for protein function or from amino acid sequence via oligonucleotides.

There are interests in comparing cDNAs from diseased and normal tissue but it should be realized that these fishing expeditions are usually unhelpful and sometimes misleading. A case in point would be the disease of cystic fibrosis, the genetic lesion for which was uncovered by linkage analysis and not by examination of 'diseased' tissue. In my view, it is unlikely that companies will undertake large-scale tissue-specific cDNA sequencing projects, largely because it is virtually impossible to know which difference to chase if and when you find it. Thus the preferred route to cDNA collection is a deterministic one. They are obtained for a particular reason: either to test expressed protein for potential therapeutic use or to be used to set up assays to look (for example) for receptor selective compounds.

How, therefore, might a random collection of cDNA sequences be of use to the drug hunters? It is possible that distant relatives of proteins with proven therapeutic utility could be uncovered. This is likely to be thrown up by sophisticated computer-driven sequence manipulation and comparison at the nucleic acid and protein level. cDNAs coding for such proteins are potentially valuable (analogous to a compound related to another compound with proven therapeutic use) but there is a long way to travel before that potential value is realized. The protein has to be expressed and characterized and its *in vivo* activity assessed well before therapeutic assessment in humans. Very much a case of a protein looking for a disease to cure.

It is equally likely that novel members of existing receptor super-families will be revealed by the same techniques, for example orphan receptors of the steroid receptor family, novel seven¹ transmembrane receptors with unknown ligands and novel 'adhesion' molecules. Once again, considerable work has to be done to uncover what the role of these proteins might be — work that would usually precede any large-scale effort to find small molecules that might affect them. Indeed, several orphan receptors exist already and there is now a hunt for the ligands. So it is the analysis of cDNA sequences that will reveal *potential* utility: much work will remain to prove it.

It is not sensible for me to debate the issue of patenting such DNA sequences, because it is essentially a legal question. Nevertheless, it is useful to remember what the actual value of cDNA sequences might be 'commercially' and what it will take to realize it.

T. J. R. HARRIS

Glaxo Group Research Limited,
Greenford, Middlesex UB6 OHE, UK

Today's non-Orwellian animal farm

Thomas H. Jukes

How is it that US inhabitants can be supplied with 218 pounds of meat annually per capita? Modern farming methods are responsible, but are unfairly criticized as inhumane.

MEAT-eating is undoubtedly a well-ingrained habit. Indeed, according to Pond¹, "the demand for animal protein increases linearly with average per capita income in all countries." Per capita annual consumption of meat in the United States in 1988 was about 72 pounds of beef, 64 pounds each of chicken and pork, and 16 pounds of turkey². Nutritional advantages include more protein in meat than in vegetable foods and higher levels of nutritionally essential amino acids in animal proteins as compared with the proteins of grains. Vitamin B₁₂ is not produced by green plants, but is present in meat, milk and eggs. Despite claims to the contrary³, 'factory farming' has not been introduced stealthily or without consultation. Actually, the new methods have been widely publicized and regulation of new procedures is delegated by Congress to the Food and Drug Administration (FDA).

Poultry have led the way. Sixty years ago poultry were generally raised in small flocks, often tended by the farmer's wife. Chickens were kept until they were so old and tough that they had to be boiled to become edible.

One of the first steps towards scientific poultry nutrition was the introduction of cod-liver oil, which supplied vitamins A and D. Ground grains such as maize, wheat, barley and oats, and grain by-products, especially wheat bran and middlings, were the main ingredients of poultry feeds. An advance was the feeding of fish meal, which had formerly been used as a fertilizer rather than a feed ingredient. Next, it was found that dairy by-products, skim milk and buttermilk, were beneficial for growth.

The 'growth factor' in milk was identified as riboflavin⁴, which was synthesized, and its price dropped from \$360 to a few cents per gram. The production of chicken broilers soon increased to a point where they consumed an amount of riboflavin greater than that in the entire milk production of the United States. The Second World War greatly reduced supplies of fish meal: soybean meal plus vitamin B₁₂ has now largely replaced it.

Another problem was the prevention of coccidiosis, an intestinal disease. Chemotherapy was the answer, first by adding sulphonamides to poultry feeds, and then by drugs selected for their action against coccidia. Another innova-

tion was antibiotic supplements to prevent chronic respiratory disease and other infectious diseases in chickens. These additions were approved for safety by the FDA.

The consumption of chicken broilers per capita in the United States was 1.1 pounds in 1937, 4 pounds in 1946 and 60 pounds in 1987 (ref. 2). The price was 21 cents per pound in 1937 and 29 cents in 1987, uncorrected for inflation. Genetics, disease prevention, nutrition and housing have contributed to increased growth and efficiency of food conversion to meat, which has been accompanied by better palatability.

Pigs have been kept to produce meat for 5,000 to 9,000 years. Under modern practices, pigs convert their food to meat at the rate of about 3.5 kilos of food per kilo of meat during growth to 100 kilos of body weight. The consumption of pork per capita has been constant for 33 years.

Modern procedures are based on prevention of the infectious, metabolic and nutritional diseases to which pigs are vulnerable. Pigs are outstanding for their consumption of maize, which must be supplemented with high-protein foods, especially soybean meal. It seems likely that essential amino acids will be supplied for pigs and chickens through recombinant DNA techniques. Applications of genetics to pig breeding and administration of growth hormone will reduce the percentage of fat in the carcass¹.

Beef cattle grow much more slowly than do poultry and pigs but cattle, because they are ruminants, can use roughage, high in cellulose, and urea, a synthetic source of nitrogen, to replace 25% of their protein requirements. Bacteria in the rumen produce B-complex vitamins, so these are not needed as additives. Beef consumption per capita has increased by about 12 per cent since 1955. Consumption of veal has decreased from 8 pounds in 1955 to only 1.4 pounds in 1988.

Productivity is an index of well-being. Economic success for intensive agriculture is measured by productivity, and even slight changes are of major financial importance. Hens stop laying eggs if they are subject to any form of adversity, including sexual harassment. In the wild state, they build nests and stop laying when a clutch of eggs has been

produced. Nidification, a genetic trait, can be induced by hormones. It has been removed by selective breeding from most hens kept for egg production. The amount of space per bird in broiler houses is critical, and is adjusted to produce maximum growth. Overcrowding reduces weight gain so treating poultry well is an economic necessity.

Chickens (and other terrestrial birds) scratch vigorously in the dirt and then look down for insects, or appear to do so. But young chickens in cages scratch the wire mesh floor and look into (and eat out of) the feed trough. Scratching is evidently instinctive rather than an 'outdoor pursuit'.

The urban public has been encouraged by the animal-rights movement to believe that animals 'feel better' when they have more space for roaming. But how do we know this is true? Humans voluntarily jam themselves together to watch sports or in social gatherings. The larger and denser the crowd, the more successful the event is deemed to be.

Opponents of modern farming methods often argue that drugs are used to prevent disease, that grains consumed by animals should be eaten by human beings instead, and that present methods are not labour-intensive. But the prevention of disease in animals leads to the prevention of suffering. The protein content of grains is low, and grains are deficient in nutritionally essential amino acids, especially lysine.

The statements that "four pounds of protein" are needed to produce one pound of protein in eggs and milk, or that more than 20 pounds of protein are needed to produce a pound of beef², are incorrect. Labour-intensive methods of animal production are arduous and unremunerative, so that few people today are willing to undertake such a task. Farm animals should be treated humanely. Their well-being is necessary for productivity. It is unfair to allege that people who are engaged in farming are oblivious to animal welfare. □

1. Pond, W. G. *Scient. Am.* **249**, 96-103 (May 1983).
2. Lasley, F. A. et al. *The US Broiler Industry* (US Dept. of Agriculture, November 1988).
3. Singer, P. *Nature* **353**, 613-614 (1991).
4. Lepkovsky, S. & Jukes, T. H. *Science* **82**, 326 (1935).

Thomas H. Jukes is a professor in the Department of Integrative Biology, University of California, Berkeley, California 94720, USA. He worked on a farm in the 1920s.

Statistical cloud over African Eden

Reconstructions of the family tree of modern people by phylogenetic analysis based on extant mitochondrial DNA appear unexpectedly difficult. An African Eden seems not yet proven.

GENESIS offers many puzzles, one of which is the appearance of entire populations from thin air at key points in the narrative. It is a cause of some wonder, for example, whence the murderous Cain recruited the inhabitants of Enoch, the city he is said to have founded after his exile, when the human population at that time had fallen sharply — from four to three. Biblical imagery, of course, has a potency that logic seems powerless to confine.

Could it be the same with science? Four years ago, the late Allan Wilson and his colleagues at the University of California at Berkeley produced a family tree of human origins based on restriction enzyme maps of mitochondrial DNA (mtDNA) from more than 130 people of diverse racial type and dispersed geographical location (Cann *et al.* *Nature* **325**, 31–36; 1987). They concluded that all human mtDNA genomes now extant derive from a single ancestral mtDNA molecule in sub-Saharan Africa about 200,000 years ago. Because mtDNA molecules are inherited maternally, the authors wrote that the ancestral mtDNA must have been present in “one woman”, by implication the ancestor of all humanity.

Inevitably, this form of words brought biblical imagery bubbling to the surface of the public consciousness. The popular evocations of Eve in her African Eden were such that one would have been forgiven for thinking that she had been interviewed in person for her comments on the *Nature* paper.

Now, the serpents have moved in. Despite a radical revision by the Berkeley group, using sequence rather than restriction data and more rigorous hypothesis testing (Vigilant *et al.*, *Science* **253**, 1503–1507; 1991), the idea of an African genesis has suffered what may be a mortally venomous bite. In a letter in last week's *Science*, Alan Templeton from Washington University, St Louis, demolishes the concept in four curt paragraphs (**255**, 737; 1992). In reply, the survivors of the Berkeley group (now all at Pennsylvania State University) succumb: Eden in Africa is unproven.

The words inscribed on the serpent's apple are “maximum parsimony”, the criterion for deciding among possible phylogenetic reconstructions by supposing that the best hypothesis has the fewest assumptions. Although it may be impossible to determine the degree to which features in related organisms are the conse-

quences of convergent evolution rather than indicative of shared common ancestry, the most parsimonious evolutionary track has a special place as a kind of lower bound to what natural selection has done. The maximum parsimony phylogeny is that inferred from data with the fewest discrete changes of character states in the course of evolution. Applied to nucleotide sequences, the most parsimonious solutions are those with the smallest numbers of base changes.

Maximum parsimony is popular for two reasons. First, character-state distributions among extant organisms suggest hypotheses about character-state distributions in even extinct ancestors (whence the enthusiasm of palaeontologists). Second, there are several desktop software packages that will generate phylogenies on these principles. In their original analysis, Cann *et al.* used a version of the program PAUP (Phylogenetic Analysis Using Parsimony).

The original data showed a greater degree of diversity among African mtDNAs (few though they were) than in any other subset of human mtDNAs classified by continent of origin. The PAUP tree reflected this in a bifurcation, one branch leading exclusively to African mtDNAs, the other to a larger subset from Africa and elsewhere. The implication was that modern human mtDNA had its home in Africa.

But neither PAUP nor any other parsimony routine can generate a unique most-parsimonious tree standing leaf-and-branch above the rest. There are probably about 7.13×10^{35} possible trees for a dataset of 134 separate items (in this case, mtDNA types) such as that compiled by Cann *et al.* Faced with such vertiginous choice, the authors concentrated on trees with an African branch, sensible enough given the independent evidence for an African origin, if over-welcoming of the conclusion.

Even that argument is incomplete. To specify geographical origin is to make geography a phylogenetic character. Last year, David R. Maddison showed that there are at least 10,000 phylogenetic trees more parsimonious than Cann *et al.*'s, most of which do not imply an African origin (*Syst. Zool.* **40**, 355–363; 1991). The Berkeley group (Wilson was still alive at the time) dismissed this analysis, citing an observation that parsimony methods neglecting sample size may produce the ‘wrong’ results (Wilson *et al.* *Syst. Zool.* **40**, 363–365, 1991). Yet the original analy-

sis could thus have been affected; the number of individuals sampled from the source population (with African mtDNAs) was much smaller than that from the supposedly derived population.

The paper from Vigilant *et al.* filled this gap, using new statistical techniques to adjust for sample size and much more detailed sampling of African populations. A PAUP analysis generated 100 equally parsimonious trees of 528 steps, themselves a selection from a number running into thousands. Nevertheless, all implied a relatively great age for African mtDNAs. Eden in African was still on the cards.

Templeton's new challenge is a double blow. First, he emphasizes that the myriad of possible trees would accommodate all possible homelands for humanity: the choice of Africa without some kind of assessment of the others is not sound. Second, he points to a procedural trap of which uninitiated users of PAUP should beware; when PAUP is used to generate phylogenies for a small number of items, the data are entered in sequence and the trees are built up as the program runs. But, Templeton says, simple sequence addition is unsuitable for large data sets (such as the sequence information compiled by Vigilant *et al.*) because the results reflect the order in which data are entered. For really big data sets, data must be entered at random and many runs performed to achieve consensus. An example shows that Africa is no more likely to have been Eden than anywhere else.

In reply (*Science* **255**, 737–739; 1992), the Penn State group concedes that its original analysis was flawed, but poses an intriguing question: what kind of data may solve the problem of human origins if an mtDNA dataset as comprehensive as that now available cannot?

The answer may simply be that the researchers were using methods inappropriate for the job. As they say, when the number of items (136 mtDNA sequences) exceeds the number of informative nucleotide positions (117), it is not surprising that the program output is so much garbage. There are 10^{267} possible trees for the latest data set: the number of maximally parsimonious trees is unknown, but is certainly “much larger than 1 billion”. Yet nobody disputes that African mtDNA is, indeed, more diverse than that from elsewhere. At least the fossils seem to indicate an African origin, even if the numbers lag behind.

Henry Gee

Repellers attract attention

John J. Sidorowich

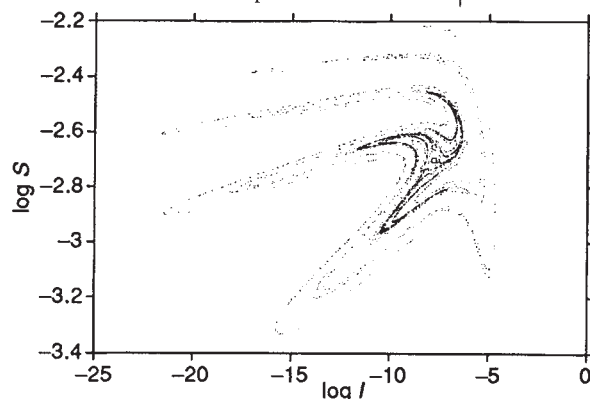
RECURRENT epidemics of childhood diseases are an important public health issue and the subject of a great deal of epidemiological research. But although knowledge of the microdynamics of infection and incubation has increased to the point where most occurrences can be cured, and in some cases even prevented, there is still much to learn about how the diseases spread through large populations. Despite speculation that chaos might play a role in the evolution of such epidemics, it has been extremely difficult to establish a coherent explanation that agrees with both theory and data. A report by D. R. Rand and H. B. Wilson in the *Proceedings of the Royal Society* (B246, 179–184; 1991) offers a fresh perspective on this issue by examining a new mechanism for chaotic behaviour in dynamical systems.

The search for chaos in the real world has been going on for nearly a decade. The most spectacular successes have occurred in fluid dynamics, where the experimentalist can generate large quantities of data under well controlled conditions. Algorithms have been developed for calculating characteristic exponents and dimensions, and methods for constructing nonlinear predictive models have been refined. But the techniques that search for some signature of chaos frequently produce ambiguous results when the data sets are small or noisy.

Such is the case for the data collected about epidemics. Instead of tens or hundreds of thousands of data points, only several hundred have been accumulated. Sampling errors can be very large, and clearly the epidemiologist can exercise little control over environmental effects. Consequently, the results from analysing the historical records are not conclusive. Several groups have tried to calculate Lyapunov exponents for the monthly records on the incidence of chickenpox and measles in major cities. The exponents characterize the sensitivity of a dynamical system to perturbations: a positive exponent indicates that small disturbances grow exponentially; a negative one that they exponentially decay. A positive Lyapunov exponent is the telltale sign of chaos, whereas if the system relaxes onto a periodic limit cycle, only negative exponents will be observed. The verdict for chickenpox is a split decision. The majority view is that there is no positive exponent, but there is contrary evidence that cannot be ignored. On the other hand, almost everyone agrees that a significant positive exponent exists for the measles data.

Confusion about the estimated expo-

nents is compounded by studies of the epidemiologists' mathematical models. It is impossible to model an epidemic on a person by person basis, so a mean field approximation is adopted where the state variables correspond to quantities averaged over a large population. A popular example is the SEIR model, a set of coupled differential equations describing the rates at which susceptible, exposed, infected and recovered (SEIR) portions of the host population grow and diminish. One of the parameters in the



A cross-sectional view of an approximation to the chaotic repeller of the SEIR equations; S , individuals susceptible to infection, I , infectives, individuals capable of transmitting the disease. (Courtesy of D. R. Rand and H. Wilson.)

equations, the contact rate, is particularly important because it is the coupling constant for the nonlinear interaction that transforms susceptibles into exposed individuals. Low values for the contact rate generate periodic limit cycles, which go through a period-doubling sequence to chaotic behaviour as the contact rate increases. There is general agreement that the value of the contact rate appropriate for chickenpox lies within the periodic range. For measles the situation is much more contentious. The SEIR model can produce a time series with the same Lyapunov exponent as the historical records, but many believe that that requires an unreasonably high value for the contact rate. However, if the value is reduced the model reverts to periodic behaviour.

An intriguing explanation for the discrepancy between the SEIR model and the historical data is provided by a new type of process named chaotic stochasticity. In their paper, Rand and Wilson point out that in addition to the periodic attractor, an unstable chaotic repeller also exists for the realistic values of the contact rate (see figure). The coexistence of these two invariant sets can generate behaviour displaying chaotic properties when perturbations are added to the system.

Attractors are invariant sets that trajectories relax onto as they evolve through the state space. The 'reduction of the state space' represented by attractors is the primary motivation behind chaotic time series analysis — the hope that what seems like an outrageously complicated dynamic could, after transients die out, result in simple behaviour that can be described in terms of an invariant geometrical object. Repellers are the unstable counterparts of the stable attractors. Points on the repeller remain there as time progresses; but points in the vicinity of the repeller, instead of being attracted onto the invariant set, are repelled away from it. Because of their instability, repellers

traditionally were not considered relevant for physical studies as they were thought to be unobservable.

Rand and Wilson investigated what happens when the mean field approximation underlying the SEIR model fails. Normally, the large number of individuals in the host population prevents the occurrence of significant fluctuations from the average behaviour. But the model equations allow for near extinctions of some subpopulations, requiring a modification in the descriptions of how these rare indi-

viduals interact with the rest of the population. As their numbers dwindle, the stochastic nature of how particular infected individuals happen to bump into susceptible folks necessarily adds a random element to the model.

Fluctuations introduced by the random interactions launch the system state into what Rand and Wilson refer to as a chaotic pinball machine. The system is knocked off its periodic limit cycle into the surrounding region of state space, which is filled with a chaotic repeller. In the absence of any further perturbations, the disturbed state would typically display a short-lived chaotic transient before relaxing back onto the periodic attractor. However, the stochastic forcing of the system does persist, and this 'noise' actually stabilizes the effects of the chaotic repeller. The chaotic transients persist as they are jostled about the fractal repeller, producing long-term trajectories with positive Lyapunov exponents.

Rand and Wilson's observations provide more than just an interesting interpretation of the SEIR model. The novel aspect of their proposal is the demonstration that chaotic repellers can act as extremely strong amplifiers for system perturbations, and that these fluctuations can stabilize the effects of the

repellor. This symbiotic relationship between deterministic and stochastic influences conspires to produce a good impersonation of a strange attractor.

Furthermore, the authors claim that chaotic repellors should be more common than chaotic attractors. If that is the case, then potential applications for these ideas will not be hard to find. The mathematical form of the SEIR equations, and the assumptions behind the use of mean field approximations, are common in many disciplines that

study complex behaviour arising from the interactions within large populations. It may be that signs of chaotic stochasticity will begin to emerge in such systems, whether they be composed of living organisms, reacting molecules, flowing particles or competing market forces. □

John J. Sidorowich is at the Institute for Nonlinear Science, University of California at San Diego, La Jolla, California, 92093-0402, USA.

COSMOLOGY

Radiation treatment for CDM

Craig J. Hogan

QUASARS are the most distant objects we see in the Universe, and their light has been travelling towards us since the Universe was less than one-sixth of its present age. In new calculations, A. Babul and S. D. M. White (*Mon. Not. R. astr. Soc.* **253**, 31P–34P; 1991) show that quasars might not only give us our best view of the early Universe, but could have influenced the development of its large-scale structure.

The most publicized embarrassment of the standard cosmological model is its failure to account for cosmic structure. But the standard model is recognized to be a huge simplification of the real world; it assumes a homogeneous distribution of matter and radiation. The only embarrassment is that cosmologists have not found the right theoretical framework for extending the model, one which catches the essential reason for the inhomogeneity of the Universe. The cold dark matter (CDM) model is one attempt at such an extension; it assumes (among other things) that structure grows by gravitational self-attraction out of primordial fluctuations dating from the inflationary epoch. The model has the virtue of making precise predictions, which is the source of its trouble: new data on the clustering of galaxies from several surveys show that the galaxy correlation function has the wrong shape, and extends to larger scales than CDM allows.

Sterilized gas

Babul and White propose a remedy for this defect in the model. They suggest that certain nongravitational processes not previously included in the model, but known to occur in the real world, might influence the formation of galaxies. Specifically, if the bright ionizing radiation around quasars somehow sterilizes gas so that it cannot form visible galaxies, the clustering of the galaxies which eventually form in the fertile, unaffected areas might match the

observed clustering, even if the quasars themselves are unclustered.

Not even strident advocates seriously imagine that the idealized mass points of CDM computer models represent the whole story of cosmic evolution, but neither is there a clear indication of what other ingredients are essential. The idea of modulating galaxy formation by the long-range influence of quasars, or of other galaxies, is not new, but the impetus to take such ideas seriously has recently increased owing to the acknowledged failure of pure CDM. The calculation presented by Babul and White is essentially statistical and geometrical, and addresses the clustering data directly. The authors use a very simple model to describe the effect of quasars on galaxy formation: each quasar creates a barren spherical region where no visible galaxies are formed.

The galaxies in the remaining unaffected regions have the same distribution as that predicted by CDM. The regions are all the same size, and the quasars are uncorrelated with each other (they pop up randomly), so the model has just two parameters in addition to CDM: the filling factor and radius of the barren regions. The interesting point is that for a plausible choice of these parameters — a choice which agrees with both the estimated space density and sphere of influence of bright quasars, and with the typical void radius in the observed galaxy distribution — the predicted correlation function of the observable galaxies matches the data on large-scale galaxy clustering.

Quasars are the brightest individual sources of light around, emitting up to 10 thousand times the light of a typical galaxy. Much of their radiation is capable of ionizing hydrogen. They are thought to play a key role in the ionization of the intergalactic (and possibly pregalactic) gas at early epochs. Because the radiation emerges from discrete sources, it is more intense near the

quasars than elsewhere. The effect of individual live quasars extends over the order of ten megaparsecs into space, which is comparable to the scale of galaxy clustering. For example, observations of intergalactic neutral hydrogen, light-absorbing clouds (the 'Lyman- α forest') show that the density of such clouds is statistically smaller in the vicinity of bright quasars out to about this distance, which is explained by the reduction of neutral-hydrogen density due to photoionization.

It could be that the clustering of visible galaxies is caused indirectly by such large-scale variations in the ionization history of the gas. The scale is about right, and the most distant observed quasars are seen at a cosmic redshift of about five, more or less contemporaneous with the earliest assembly of some of this pre-galactic gas into protogalactic clouds in the CDM model.

Sphere of influence

The general idea is also plausible on physical grounds. Quasars are relatively short-lived; although at any given time only a small fraction of space is within the ionizing sphere of influence of a bright quasar, much of space comes into such a domain at some point in time, where the total ionizing field is dominated by the nearest single source. Possibly, ionizing radiation could have a permanent effect on the subsequent evolution of the gas — for example, it destroys coolants such as molecular hydrogen, which are difficult to regenerate at such low densities.

Gravitational collapse of gas is regulated by the rate at which binding energy can be radiated, which is why stellar nurseries in our own Galaxy are dense regions shielded from the external sources of ionization that destroy coolants. Thus, intense radiation might make it more difficult for protogalactic gas to cool and form a visible galaxy, or perhaps it leads to the formation of stars of lower mass which are harder to see. Either way, the cumulative effect of quasar radiation might alter the clustering of visible galaxies; those fertile protogalaxies lying outside the huge ionizing spheres of influence around quasars will have their relative correlations with each other increased, on a scale comparable to the size of these regions.

It is certainly suggestive that a known population of radiating sources has both the potential to affect galaxy formation and roughly the geometry needed to produce the galaxy correlations we observe. However, it is worth listing several potential difficulties with the model. First, the mechanism modulates only the galaxy density, not the mass density; the voids are still full of barren or dark material. Therefore, the model

cannot explain observations of large-scale flows which appear to confirm the existence of mass fluctuations matching the galaxy clustering. Also, the galaxy voids today should be full of 'failed galaxies', but this population has not been detected, even in the nearby voids. (Or has it? There are some very tenuous galaxies, and many faint and weakly clustered ones, being discovered.)

If CDM is still retained as the authors prefer, there even ought to be 'dark galaxy clusters' in the voids, whose X-ray emission should be detectable, but is not seen. At the relatively small redshifts (of the order of 1, far smaller than 3–5 where sterilization takes place) where galaxies and quasars are both detected, there is a positive correlation between the populations, not an anticorrelation. Faint galaxies show much weaker correlations than would be expected if they were young galaxies already distributed in large-scale structures.

There is a disturbing coincidence in the best-fitting model parameters that the quasar influence must include most, but not quite all, of space. The Lyman-limit and C IV (ionized carbon) absorption systems which are thought to be associated with primaeval galaxies show no sign of avoiding live high-redshift quasars, the way that the Lyman- α absorbers do (although here the data are skimpy and may yet provide support for the scheme). And there is not enough known about the details of galaxy formation to say for sure whether the modulation of galaxy brightness by ionization history really works.

Sceptics will be unimpressed by the agreement with the clustering data in a model which adds two more free parameters. But the overall message represents a clear maturing of cosmological science, a progress into more realism forced by the data. I can't summarize the bottom line more eloquently than Babul and White: "... the modulation of galaxy formation by non-gravitational processes could, in principle, be responsible for much of the apparent large-scale structure. This possibility should therefore be considered carefully, and eliminated, before the observed structure is used to draw conclusions about the physics of the early Universe and the mass fluctuations which it produced". At present, there is no reason to think this possibility will be eliminated. Perhaps it will seem incredible to future cosmologists that people ever tried to separate the evolution of galaxy formation and clustering from the thermal and ionization history of the pregalactic gas. We still do not understand how these two grand stories are related to each other.

Even if it works, this kind of medicine for CDM may be strong enough to kill the patient. If a nongravitational

mechanism plays a central role in creating the largest-scale structure — the phenomenon CDM was uniquely positioned to explain — then it is natural to suppose that the same is true of structure on smaller scales. Even though gravity alone controls the orbits of galaxies today, the direct gravitational effects of truly primordial fluctuations could be

negligible; everything we see, aside from the structureless uniformity on the very largest cosmic scales, could be the result of emergent structures created by evolutionary astrophysical processes. □

Craig J. Hogan is in the Department of Physics, University of Washington, Seattle, Washington 98195, USA.

CHEMICAL BONDING

Fixing a molecular snapshot

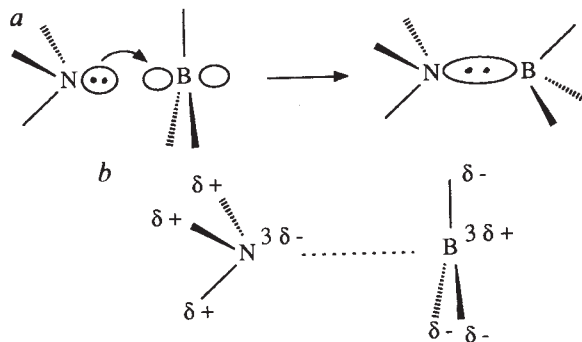
Patrick Fowler

A CHEMIST can expect to guess with fair accuracy the bond lengths in a new molecule by summing empirical atomic radii. New work by Dvorak *et al.*¹ on the microwave spectrum of the gas-phase complex between acetonitrile (CH_3CN) and boron trifluoride (BF_3) will worry users of this simple approach. In similar compounds the B–N distance is either about 1.6 Å for a covalent bond, or about 2.7 Å for a weak van der Waals bond. By contrast, the newly measured

B–N bonding orbital by overlap of the highest occupied molecular orbital of NH_3 and the lowest unoccupied molecular orbital of BX_3 .

Definite structural consequences follow — a short B–N bond length and tetrahedral geometry about boron. Both features are found in the crystal structures of compounds H_3NBF_3 , $(\text{CH}_3)_3\text{NBF}_3$, $(\text{CH}_3)_2\text{NBF}_3$, and also in the solid acetonitrile adduct. The classic Lewis example, H_3NBF_3 , has also been studied in the gas phase⁴.

The B–N distance and F–B–F angle have correlated effects on the spectrum, but reasonable assumptions lead to a distance hardly changed from the crystal value; low nuclear quadrupole coupling constants suggest significant pyramidization at B, again as in the crystal. Similar results might have been expected for gas-phase $\text{CH}_3\text{CN}\cdots\text{BF}_3$, but barring unusual dynamic effects, the rota-



Contrasting models of the boron–nitrogen bond: a, covalent adduct; b, long-range attraction.

distance in gas-phase $\text{CH}_3\text{CN}\cdots\text{BF}_3$ is 2.011 Å, squarely between the two bonding types and 25 per cent longer than in crystals of the same compound. Although apparently unprecedented in experiment, an intermediate bond length had been predicted for this molecule by *ab initio* calculations² that were used to guide the search for the spectrum.

Puzzling over boron compounds has been a fruitful source of new ideas several times in the history of chemistry. In the early part of this century, the anomaly of boron-containing adducts was resolved when Lewis formulated his electron-pair theory of the chemical bond³. Boron trialkyls and trifluoride react with ammonia and other bases to form crystalline compounds. This reaction of two apparently saturated compounds was rationalized in terms of a dative bond, by which nitrogen donates a share of its unbonded lone pair of electrons to complete the unfilled octet of electrons in boron. In more modern language, the two combine to form a

tional constants obtained for various isotopomers¹ imply a longer bond and near planar BF_3 .

Ironically, when Lewis was formulating the concept of the two-centre, two-electron bond, the boron compounds that would prove it incomplete were being synthesized. The boron hydrides broke all the rules of valence, and chemists' preoccupation with these 'electron-deficient' structures spawned the modern qualitative molecular-orbital theory of bonding in main-group and transition-metal clusters.

A third kind of boron compound then appeared⁵. In a molecular-beam gas expansion, weakly bound dimers can survive long enough for structural characterization, and the technique has been used for scores of pairs. Early complexes involving the B $\cdots$ N link included $\text{N}_2\cdots\text{BF}_3$ and $\text{NCCN}\cdots\text{BF}_3$, with $\text{HCN}\cdots\text{BF}_3$ being added to the list more recently. The spectra are consistent with B–N distances in excess of 2.5 Å and a planar BF_3 , differing qualitatively from

the classical strong adduct.

Weak dimers are usually described as associations of unperturbed monomers under the influence of long-range (mainly electrostatic and dispersion) attraction, held from collapse by short-range repulsion (the van der Waals envelope). Although the empty coordination site of BF_3 gives pause for thought, electrostatic interaction of the significant molecular quadrupole of BF_3 with the dipole or quadruple of the incoming base would account for electric properties and structures of the weaker complexes.

Dvorak *et al.*¹ use an idea from solid-state studies⁶ and interpret the pairs of B–N distances R and N–B–F angles α for different complexes as snapshots along a general reaction path for nucleophilic attack of N on B. Nonpolar N_2 and NCCN give weak complexes with long bonds and represent the tentative approach at the start of the reaction; with dipolar NH_3 the stronger attraction pulls the monomers into close contact and the full-blown charge-transfer adduct forms. The dipolar Lewis bases give rise to the crystallizable complexes. In some cases the reaction can proceed further, to form an even shorter B=N double bond with elimination of HF.

Position on the reaction coordinate is correlated with an independent measure of nucleophilicity derived from hydrogen-bonded complexes⁷ which ranks the bases $\text{N}_2 < \text{HCN} < \text{CH}_3\text{CN} < \text{H}_3\text{N} < (\text{CH}_3)_3\text{N}$. Overall dipole is a poorer measure; acetonitrile has a larger dipole than either HCN or ammonia and yet its complex lies between them on the (R, α) curve. The large moment is, however, probably the reason for the anomalously large shift in R from gas to solid, as a lattice of polar dimers would stabilize charge separation in the same way as the Madelung potential in an ionic solid. So, it seems to be the combination of moderate nucleophilicity with large dipole moment that makes the new complex odd. It would be interesting to know where solid $\text{HCN} \cdots \text{BF}_3$ falls on the curve (presumably also at low R), and whether by tinkering with substituents on the base, more molecules can be caught in the act of bond formation. □

Patrick Fowler is in the Department of Chemistry, University of Exeter, Stocker Road, Exeter EX4 4QD, UK.

Cell cycle arrest and *c-mos*

Tim Hunt

THE *mos* oncogene encodes a serine-threonine protein kinase and is normally expressed only in germ-line tissues. Indeed, translation of maternal *c-mos* messenger RNA is necessary for *Xenopus* oocyte maturation¹. Recent attention has focused on the surprising evidence that *c-mos* protein is a component of cytotostatic factor (CSF), the entity that stabilizes maturation-promoting factor (MPF) and maintains meiosis II metaphase arrest in frog eggs until the sperm arrives². New data from Yew *et al.* on page 649 of this issue³ shift the emphasis back to the ability of *c-mos* protein to promote oocyte maturation and shed light on its interaction with progesterone, the natural stimulus of oocyte maturation. These aspects of its action are probably more relevant to the transforming properties of *c-mos* protein than its connection with CSF, for it is not obvious how a kinase that arrests the cell cycle in eggs can promote cell-cycle progression when introduced into normal somatic cells.

The new results were made possible by constructing a fusion gene between the *Escherichia coli* maltose-binding protein (MBP) gene and *Xenopus c-mos*, which enabled the production of a soluble MBP–Mos fusion polypeptide that became active as a protein kinase after its introduction into oocytes. It had previously been shown that injection of both *c-* and *v-mos* mRNA could promote oocyte maturation in the absence of progesterone, but fairly large amounts were needed, and the efficiency of maturation tended to fall short of 100 per cent^{4,5}. There was a troublesome paradox, too, with regard to the CSF properties of *c-mos* protein, for it was hard to understand why, if *c-mos* could make oocytes pass from G2 into meiosis metaphase I, the oocytes did not arrest then and there, instead of moving on to meiosis II (ref. 6). Either something else besides *c-mos* protein was needed to stabilize MPF, or perhaps a special protease destroyed *c-mos* protein at the end of meiosis I.

When Yew *et al.* injected MBP–Mos into stage VI frog oocytes they matured, whereas in cleaving embryos MBP–Mos arrested the injected blastomeres in metaphase, just as expected from the previous mRNA injection experiments. Alteration of Lys 90 to Arg abolished the kinase activity of MBP–Mos, and this construct had no biological activity. So far, so good, but nothing new. The next experiment was to test if the maturation-promoting activity of MBP–Mos was retained when protein synthesis

was blocked by cycloheximide. An important property of MPF, as compared with progesterone, is its ability to promote maturation without the need for new protein synthesis⁷. Is the synthesis of new *c-mos* all it takes to trigger the G2–M transition in oocytes? Curiously, it turned out that cycloheximide reduced, but did not abolish, the MPF-like property of MBP–Mos. A lower fraction of oocytes matured, and considerably higher levels of the fusion protein were needed compared to the endogenous levels of *c-mos* protein found in *Xenopus* eggs. The striking result was that progesterone greatly increased the potency of MBP–Mos in the presence of cycloheximide. Thus, in the absence of protein synthesis, progesterone alone had no effect, and neither did a low dose (2.7 ng) of MBP–Mos. Add both together, however, and almost all the oocytes matured.

This is a very revealing finding, because it shows that progesterone does more than just turn on new protein synthesis; it must also remove some inhibitor(s) of cell-cycle progression. A plausible candidate for this inhibitor is cyclic AMP-dependent protein kinase (A-kinase), considering the old findings of Maller and Krebs, who were able to promote oocyte maturation by introducing inhibitors of A-kinase, and conversely, inhibited progesterone-induced maturation with A-kinase catalytic subunit⁸. It makes good sense that progesterone normally acts by both releasing the brakes and stepping on the accelerator, which is what the results of Yew *et al.* clearly show.

But this is not all. What about the paradoxical lack of meiosis I arrest mentioned earlier? Do the MBP–Mos-injected oocytes reach meiosis I or II? The answer is that they do not make it to meiosis II; new protein synthesis is required for that. The new protein(s) cannot be replaced by a fresh shot of MBP–Mos, and in fact, MBP–Mos is perfectly stable after meiosis I, which disposes of the idea that failure to arrest at meiosis I was due to instability of *c-mos* protein at this juncture. It is also unlikely that new cyclin synthesis accounts for the protein synthesis requirement for entry into second meiotic metaphase, for although some of the maternal B-type cyclins are lost by proteolysis at meiosis I, enough survive (it seems) to allow the return of MPF (refs 9, 10). So, it looks as if the product of at least one more important (perhaps unknown, perhaps not) maternal mRNA must contribute to the second rise in

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MPF, and presumably collaborates in some way with *c-mos* to stabilize MPF until the coming of the sperm. It will be fascinating to learn what it is.

These simple and elegant data make it much easier to understand the transforming ability of *c-mos*, because they show that it does not cause metaphase arrest by itself, whereas *c-mos* alone strongly promotes oocyte maturation. But what does oocyte maturation have to do with transformation? More than you might suppose, in my opinion. Although it is true that oocytes are special, poised on the brink of a unique process in the life of the organism (meiosis), their state has much in common with other kinds of resting cell. They are in suspended animation as far as the cell cycle is concerned, and need a concerted programme of translational and post-translational changes to release them from this state. It is appropriate to regard them as being in the G2 equivalent of G0, which, alas, has no official name. In support of this view, I adduce the changes in protein kinase activity that accompany oocyte activation, which are strongly reminiscent of those in serum-stimulated fibroblasts. For example, both MAP kinase and S6 kinase are activated at about the same time that MPF activity first appears^{11,12}.

It is not yet clear what specific role *c-mos* protein has in this process, but for the time being, Yew *et al.*'s results suggest that *c-mos* protein should be viewed as a protein kinase that helps cells escape from dormancy. Thus, its introduction into somatic cells probably makes it more difficult, if not impossible, for them to enter G0. This provides a satisfactory general explanation for the extraordinary potency of this oncogene, if short on molecular details. Anyway, better forget about the CSF business for now, until the mystery protein(s) have been identified. The full story has yet to be told, and more important lessons about cell-cycle transitions will surely be learned from *c-mos*. □

Tim Hunt is at the ICRF Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK.

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Amazon rocks the cradle

Paul G. Bahn

A REPORT in *Science*¹ by Anna Roosevelt and colleagues describes the unearthing on the lower Amazon, Brazil, of the oldest known pottery in the Western Hemisphere. The influence of this new work will be considerable, for it draws attention to other, long-neglected evidence that the tropical lowlands, rather than the Andes, were the cradle of civilization in South America.

Very little professional archaeological work has ever been carried out in

region also has large areas of alluvial soils — as fertile as the flood plains of the Nile, Ganges and other great rivers — with a riverine seasonal forest and savanna that are rich in fish, game and plants. Between 1830 and 1945 evidence had already accumulated that this region had deep, stratified middens, earthworks, elaborate pottery and other artefacts. Some middens were assigned to the early Holocene by nineteenth-century researchers on geological



Europeans arrive in Brazil, with predictable consequences for the indigenous population.

Amazonia. Before the advent of antibiotics and immunization, the area could be lethal; the few scholars who worked there often died young, and their discoveries remained unpublished or were largely ignored. In the twentieth century, on the other hand, the vast majority of archaeological research in South America has been directed to other areas. Environmental determinists claimed that tropical rainforest would have prevented any substantial prehistoric human occupation or long-term cultural development in the lowlands. It was assumed, and became dogma, that the Andes were the centre of cultural development, and that horticulture was brought to the lowlands by intruders from the Andes who displaced the region's population of hunter-gatherers. The latter, it was thought, had been unable to develop early agriculture, ceramics or complex societies for themselves because of the poverty of tropical resources.

This traditional view ignored environmental and archaeological evidence to the contrary. Amazonia may have resource-poor rainforests, but this vast

grounds. But more recently it was generally assumed that this material was no more than 1,400 years old. Only a few pioneers such as Donald Lathrap² continued to argue for Amazonia's chronological priority and cultural complexity.

In 1987, excavations were carried out by Roosevelt and colleagues at the prehistoric shell-midden of Taperinha, near Santarém, Brazil¹. This mound, over 6 m tall and covering several hectares, lies on the edge of an ancient terrace of the lower Amazon. The excavations exposed 48 strata of freshwater mussel-shells, charcoal and animal bones together with 383 fragile potsherds and a few human bones. Radiocarbon dates (using accelerator mass spectrometry) were obtained from four samples of charcoal, five of shell and two of elemental carbon and humic/fulvic acids from the base of a pot: the results, when calibrated, spanned the period from 8,050 to 6,950 years ago. In confirmation, thermoluminescence dating of a fragment of the same pottery gave a result of 7,110 ± 1,422 years ago.

The Taperinha pottery, grit-tempered and brown-red, some of it decorated

BLACK HOLES

Placing faith in the masses?

Joseph F. Dolan

with painted patterns or with an incised rim, is therefore at least a thousand years older than the previous earliest ceramics of South America, from fourth millennium BC Colombia^{3,4}, and 3,000 years older than the first pottery in the Andes or Mesoamerica.

The upper part of the region's shell-mounds contains numerous grinding stones which may reflect incipient root horticulture. From about 4,000 years ago, there were ceramic-stage villages with manioc horticulture, two millennia before the central Andes had reached that stage. Roosevelt argues that the broad Amazon floodplain may have been one of the most densely inhabited zones in the prehistoric New World: ancient middens are almost continuous along the banks of the lower Amazon, often several metres deep, and some areas of the floodplain are densely covered with earthworks over hundreds of square kilometres. By about 2,000 years ago there were populous agricultural chiefdoms, both there and in the upper Amazon, which produced settlements of apparently urban scale and complexity.

Other investigations⁵ on the immense Marajo Island at the mouth of the Amazon, have revealed the existence of a complex society of the fifth to the fourteenth centuries AD, featuring monumental earthen mounds, cemeteries of elaborate funerary urns and a rich material culture with complex and varied art styles. Although there are no obvious images of chiefs, this was probably a ranked or stratified society, one based not on intensive agriculture but on a mixed diet, primarily seeds and fish. Indeed, from analysis of the human skeletal remains it seems that its people enjoyed better living conditions than many modern Amazonians.

The Europeans who came to the region in the seventeenth century found a large indigenous population, one led by chieftains and supported by agriculture, foraging, trade and tribute. Unfortunately the Europeans fought and defeated them to make way for their vast ranches, and by 1850 or so the native population had disappeared. Only archaeology can hope to resurrect this culture, which may hold the key to the origins of many of South America's cultural achievements. □

Paul G. Bahn, 428 Anlaby Road, Hull HU3 6QP, UK, is a freelance writer on archaeology.

BECAUSE black holes require both quantum mechanics and general relativity to describe them completely, a reliably identified black hole would provide physicists with a useful testbed on which to verify and refine attempts to unify the two theories. Computed evolutionary tracks of massive stars lead them to a black-hole state at the end of their lifetime, so X-ray binary systems (one of whose constituent pair is a collapsed star responsible for the X-radiation) provide the likeliest stellar elephants' graveyard in which to search for a black hole. The new results of Casares, Charles and Naylor, reported on page 614 of this issue¹, provide strong evidence that the primary object in the X-ray transient system V404 Cygni is in fact a black hole.

The basic problem encountered in finding a stellar black hole is that of identifying an object hidden forever behind its encircling event horizon. The currently favoured method of identification — the one used by Casares, Charles and Naylor — derives the mass of the unseen X-ray source from spectroscopic observations of radial velocity variations in the system. The spectral lines observed originate in material outside the event horizon, either in the companion star or in a disk of material being dragged into the hole. The resulting spectroscopic mass function is then the minimum possible mass for the X-ray source. Knowledge of the mass of the visible star (estimated from its apparent spectral type) and the inclination of the binary orbit to our line of sight (generally derived from measurements of variable polarization in the system) can then provide a specific value for the mass rather than a lower limit.

The X-rays are produced by the conversion into radiation of the kinetic energy gained by mass outflowing from the secondary and falling towards the unseen star. The observed X-ray luminosity demands a gravitational potential well so strong that the object's mass must be confined to a region of space with characteristic size of a few tens of kilometres. Hence, the argument goes, the object must be compact, so small that the principal force opposing its gravitational collapse is the degeneracy pressure arising from the quantum-mechanical exclusion principle. The lower limit of 6.3 solar masses that Casares, Charles and Naylor report for V404 Cyg ties it with Cyg X-1 as the mass champion² of collapsed objects. (Stars still burning can be more massive, of course.)

By the Rhoades–Ruffini theorem³, the maximum theoretical mass of any compact object that is not a black hole is about three to five times the mass of the Sun. In essence, if one assumes only that the mass-energy density in the object is positive definite, and that local microstability and the laws of causality exist everywhere in the object, then one can show that the maximum mass of a uniformly rotating compact object with the stiffest known equation of state which is not a black hole is 3.2 solar masses; any more and the material cannot resist the crushing weight of its own gravity. If the constraint of causality is relaxed (this ultimately changes the relation between temperature and pressure), then the maximum theoretical mass of a non-rotating uniform density sphere is 5 solar masses⁴. By this line of argument, V404 Cyg must be a black hole.

The upper mass limits from the Rhoades–Ruffini theorem are derived by matching a model of a stellar core, in which any equation of state might be valid, to an envelope in which the equation of state is assumed to be both known and unique. The resulting masses depend on ρ_0 , the density at the boundary between the core and the envelope⁵. For ordinary hadronic matter, ρ_0 is usually assumed to be about 10^{14} g cm⁻³, a density far above any attained experimentally. The resulting model represents a neutron star. If some different equation of state were valid above densities significantly less than 10^{14} g cm⁻³, then the model's core would have to be correspondingly larger, and the mass limits derived from the Rhoades–Ruffini theorem would be significantly greater than those derived for neutron stars.

Recent investigations into the equation of state at nuclear densities have discovered an equation of state different from those describing a neutron star but consistent with all known nuclear physics data and the standard model of particle physics⁶. In this equation of state, high-density baryonic matter is bound primarily by the strong nuclear force rather than gravity, so objects more massive than neutron stars can be stable against gravitational collapse by assuming a lower density than a neutron star. The models using this equation of state represent objects called Q stars.

Even if one dismisses Q stars as being merely hypothetical, the possibility remains that some equation of state other than that describing neutron stars may contain the correct physics at nuclear densities. The scale density ρ_0 may then be much less than 10^{14} g cm⁻³. White-

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dwarf stars, being correctly described by the ordinary equation of state, give us the next best limit on ρ_0 , 10^{10} g cm $^{-3}$; this lower value of ρ_0 allows Q-star models to be constructed with masses well above those of neutron stars⁷. Unless the Q-star equation of state can be shown to be invalid below nuclear densities, it is not logically necessary that a compact object with a mass above 5 solar masses must be a black hole.

The end result seems to be that although massive compact objects in X-ray binaries, such as V404 Cyg and Cyg X-1, cannot be classical neutron stars, proving that they are black holes will require the detection of some phenomenon unique to a black hole other than its total mass. One possibility is the observation of dying pulse trains⁸. These strings of millisecond pulses, decreasing in both separation and intensity with increasing pulse number, originate inside the last closed orbit around a black hole,

from luminous clumps of matter executing their final few revolutions before reaching the event horizon. The High Speed Photometer team of the Hubble Telescope will be attempting to detect such dying pulse trains from black-hole candidates in the near future. □

Joseph F. Dolan is in the Laboratory for Astronomy and Solar Physics, NASA Goddard Space Flight Center, Greenbelt, Maryland 20771, USA.

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HUMAN GENETICS

Disease and development

Michael A. Walter and Peter N. Goodfellow

It is an expectation amongst developmental biologists that mutations in the genes that regulate development should contribute to human genetic disease. This simple hypothesis has not received overwhelming support, despite the identification of several important families of regulatory genes. Investigation of the Pax gene family, however, is beginning to reveal the molecular basis of neuronal and muscular disease in both mice and humans^{1–3}. The *Splootch* phenotype in the mouse affects structures derived from the neural crest and is caused by mutation in the embryonically expressed *Pax-3* gene⁴. On pages 635 and 637 of this issue^{5,6}, two groups demonstrate that Waardenburg's syndrome, a human disease, results from mutations in *HuP2*, the homologue of the mouse *Pax-3* gene.

Neural crest

Waardenburg's syndrome is a hereditary disease that is responsible for over two per cent of cases of adult deafness. The syndrome has been divided into two clinical forms, type 1 and type 2, both of which are inherited as autosomal dominant diseases. Type 1 is characterized by deafness, a disturbance in pigmentation (often expressed as a frontal white blaze of hair and/or white eyelashes), and lateral displacement of the inner corner of each eye⁷; the eye displacement is absent in patients suffering from the type-2 disease. The tissues affected in Waardenburg's syndrome are related by their embryonic origin, and it has been suggested that the disease is caused by a

developmental defect in the neural crest⁸.

The mouse and human Pax genes were initially cloned based upon their homology to the *Drosophila melanogaster* genes *paired* and *gooseberry*, which are in part responsible for segmentation and segment polarity. Eight different murine and three different human Pax genes have been described, all of them sharing a conserved sequence motif, the paired box, which encodes a DNA-binding motif⁹. By analogy with *Drosophila*, it is assumed that at least some members of the Pax gene family are involved in embryogenesis, and defects in three Pax genes have been shown to cause developmental abnormalities. The mouse *undulated* phenotype is the result of mutations in *Pax-1* (ref. 1); the mouse *small eye* and human aniridia disorders are due to mutations in *Pax-6* genes^{2,3}; and *Pax-3* mutations cause the *Splootch* phenotype⁴. *Splootch* mutant mice are characterized by spina bifida, and dysgenesis of neural crest cell-derived spinal ganglia and heart structures. In addition, *Splootch* mutants have white spots on the abdomen, tail and feet.

The expression of *Pax-3* is first detected in 8.5-day mouse embryos and is localized to the spinal cord and regions of the hind and midbrain during early neurogenesis¹⁰. The gene encodes a protein with a paired box domain and a paired-type homeodomain, both of which are involved in DNA binding. When *Pax-3* was mapped to the proximal part of chromosome 1, the map

position and coincident tissue distribution of *Pax-3* transcripts with the tissues affected in *Splootch* mutants led to investigation of the *Pax-3* locus in *Splootch* mice⁴; the identification of a 32-base-pair deletion in *Pax-3* confirmed that *Splootch* was a mutation at this locus.

Type-1 Waardenburg's syndrome was first mapped to human chromosome 2 by linkage to placental alkaline phosphatase. Further refinement of the map position was based on a young Waardenburg's patient with a chromosomal inversion of part of the long arm of human chromosome 2. The inverted region is homologous to the proximal part of mouse chromosome 1 where the *Splootch* gene is located. From the overlapping phenotypes and related map locations of the type-1 disorder and *Splootch*, it seemed that these syndromes could be the result of defects in homologous genes.

Altered control

Tassabehji *et al.*⁵ and Baldwin *et al.*⁶ tested this relationship by analysing *HuP2* in families prone to Waardenburg's syndrome. *HuP2* is a human gene that is identical in amino-acid sequence to *Pax-3* over amino acids 2–121 of the paired domain. Both the deletion of six amino acids found in one family by Tassabehji *et al.*, and the alteration of a proline to a leucine residue identified in a large Brazilian kindred by Baldwin *et al.*, are mutations in the paired domain of *HuP2*. The substitution of proline to leucine occurs at a position within the paired domain that is invariant in Pax proteins¹¹. As the *Pax-3/HuP2* paired domain is known to bind DNA, it is not beyond the bounds of possibility that these mutations result in the altered control of expression of genes involved in early neurogenesis.

As well as contributing to our understanding of the control of early neural development, the discovery of the molecular basis of the Waardenburg phenotype is of practical importance. The *Splootch* mouse now becomes a model for Waardenburg's syndrome, and this in turn may suggest that an investigation of *Pax-3* in spina bifida and other neural tube defects would be

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worthwhile. It has been proposed that deafness in the white cat is a model for Waardenburg's syndrome, a claim which can now be tested directly by analysis of the cat *Pax-3* gene.

Non-geneticists are not always convinced of the importance of genetic maps. This attitude is mistaken for, as the new work again shows, such maps allow the association of phenotype with specific genes — the *Pax-Splotch* and

the *Splotch*-Waardenburg type-1 connections both depended on having good genetic maps. As the maps become more detailed, the connections will become more obvious, thereby emphasizing the relationship between disease, development and genes. □

Michael A. Walter and Peter N. Goodfellow are in the ICRF Laboratories, Lincoln's Inn Fields, London WC2A 3PX, UK.

QUANTUM MECHANICS

Bringing order out of chaos

R. V. Jensen

A LOT of deep thought has been stimulated by the apparent contradiction that probabilistic quantum mechanics can be more regular and predictable than the corresponding deterministic, but potentially chaotic, classical mechanics. The first experimental test of the ideas invoked to resolve this paradox is reported by S. Sridhar¹, who used microwaves as analogues of quantum-mechanical matter waves to show that the 'scarred wavefunctions' supposed to reconcile these two views² really do exist. And G. S. Ezra *et al.* show³ that these scarred wavefunctions can solve one of the oldest problems in pre-quantum-mechanical Bohr theory — the structure of the helium atom.

Two classes of classical mechanical systems that exhibit chaotic behaviour are represented by atoms with a single

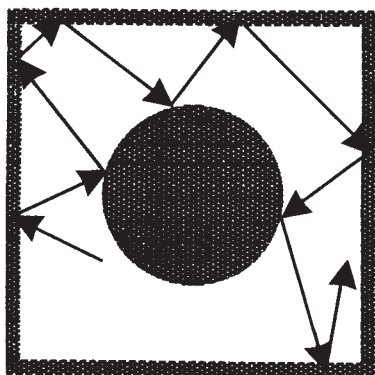


FIG. 1 The chaotic classical motion of a ray or particle in a square Sinai billiard is chaotic.

highly excited electron and by the helium atom. In the first, any small perturbation should send the excited electron's path astray; in the second, the strong coupling between helium's two electrons should disrupt any order imposed by the central positive nucleus. Yet quantum spectroscopy shows both systems to be remarkably regular.

The term scarred wavefunction was first coined to describe the surprisingly regular structure of the computed eigenfunctions for a free particle moving in a

bounded domain shaped like a stadium², intended as a crude, tractable model for complex atoms. The classical motion of a particle bouncing around this frictionless billiard table is fully chaotic. A typical trajectory will uniformly cover the domain's surface, given enough time.

Some trajectories make closed loops, so that a particle might repeat its path. But although these classical paths are periodic, they are highly unstable: a small perturbation, no matter how small, will divert the trajectory from its repetitive path. The surprise is that when the particle's behaviour is examined using Schrödinger's wave mechanics, many of the wavefunctions appear to be strongly peaked along the short, unstable periodic orbits, indicating regular behaviour. Although these scarred wavefunctions have been indirectly shown to play an important role in the experimental studies of atoms in strong fields⁴, Sridhar's direct experimental measurements of scarred wavefunctions in an electromagnetic cavity provide a graphic demonstration of the ubiquity of this phenomenon.

The quantum eigenstates of a particle in a bounded domain, like the resonance modes of an electromagnetic cavity, are solutions of wave equations. Exploiting this analogy, Sridhar constructed an electromagnetic cavity with a two-dimensional cross-section shaped like a rectangle surrounding a circular disk — the so-called Sinai billiard (Fig. 1). In this case the ray dynamics of geometric optics are analogous to the classical motion of a particle in this domain, and the electromagnetic waves are equivalent to the quantum mechanical ones.

Sridhar mapped out the resonance wavefunctions by moving a small metallic bead around the cavity. As the bead approached a maximum of the electric field, the resonant frequency shifted by an amount proportional to the local field strength. By moving the bead around using magnetic forces and measuring the shifts in resonance frequency, Sridhar generated an experimental snapshot of

each resonance wavefunction (Fig. 2), showing that the wavefunctions are peaked along the short unstable periodic orbits.

Not all of the eigenfunctions exhibit such visible scars. But many do. For the numerically calculated eigenfunctions of the stadium, up to half of the eigenstates at the level of the 10,000th excited state seem to be peaked along one or more periodic orbits. The Sinai billiard has relatively fewer identifiable scars because of the greater instability of the short periodic orbits.

The remarkable regularity of some wavefunctions for classically chaotic sys-

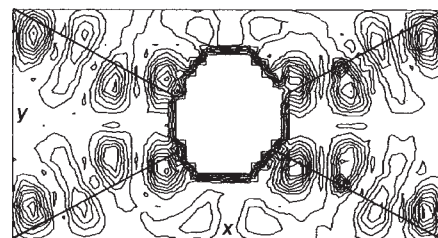


FIG. 2 Sridhar's experimental measurements were performed in a microwave cavity with a cross-section of a rectangular Sinai Billiard. This topographic map of the measured electric field strength for a resonance mode of this cavity clearly exhibits scarring along the diagonal, classical periodic orbits.

tems has far-reaching implications. Statistical theories based on the ergodicity of the chaotic classical dynamics for the equilibration of energy in excited molecules may be strongly modified by quantum effects⁴. The behaviour of atoms in strong fields, and of ballistic electrons in small, mesoscopic solid-state devices, may be similarly affected. Although the classical motion may explore all the available phase space, the quantum evolution for scarred states remains highly localized.

Although there is no comprehensive theory for this phenomenon, considerable progress has been made using elaborate semiclassical theories based on the Feynman path-integral and the Van Vleck formulations of quantum mechanics. Gutzwiller⁵ showed in the 1970s how the unstable, periodic orbits of chaotic classical systems can be used to approximate the quantum-mechanical Green's function, which contains information about the distribution of energy levels and the structure of the eigenfunctions. Although we are still unable to predict when specific wavefunctions will be scarred by particular orbits or even which systems or energy ranges will be more heavily scarred, these semiclassical theories describe the regular modulations of the absorption spectra of atoms in strong magnetic fields⁶.

The most significant application of these new ideas is in the first successful semiclassical quantization of the classi-

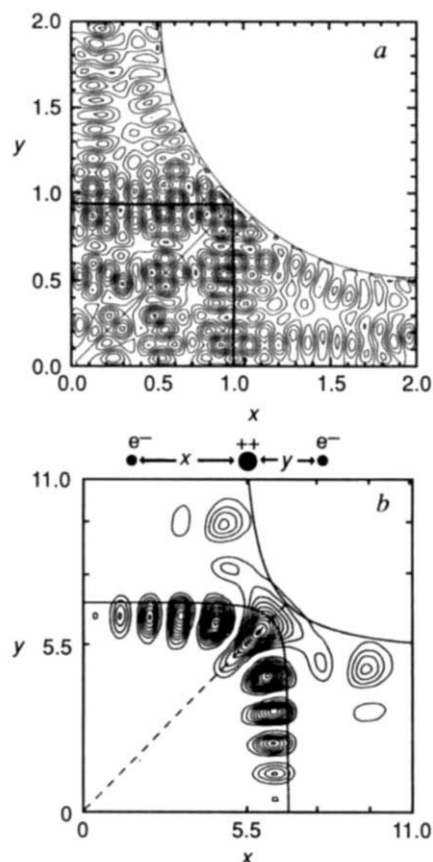


FIG. 3 *a*, A topographic map of a numerically calculated eigenstate in the square Sinai billiard with a scar along the right-angle periodic orbit. Because this trajectory collides twice with the circular scatterer each period, it is more unstable than the diagonal periodic orbits and the associated scars are less distinct than the diagonal scar shown in Fig. 2. (The computer code for this calculation was provided by E. Heller.) *b*, A topographic map of a numerically calculated wavefunction for a doubly excited state with quantum numbers $n = 6$, $L = 0$. The wavefunction is clearly peaked along the right-angle periodic orbit.

cally chaotic helium atom, reported by Ezra *et al.*³. The inability of the Bohr-Sommerfeld semiclassical quantization prescription to calculate the energy levels for the helium atom led to the downfall of the old quantum theory in the 1920s. In this, the invariant classical actions were associated with integer quantum numbers multiplied by Planck's constant. But nonintegrable (chaotic) classical systems may have no good action variables to quantize, as recognized in a little known paper⁷ by Einstein in 1917.

The classical description of the electrons in a helium atom is a nonintegrable three-body Coulomb problem, so that the old theory failed to determine the energy levels. The new evidence of the role of unstable periodic orbits in such situations suggests a way forward — especially for the doubly excited states that are difficult to describe even with modern quantum mechanics, because of

strong electron-electron correlations.

Ezra *et al.* consider a collinear model of the helium atom, with the electrons on either side of the nucleus. The classical configuration space for this simplified model consists of the two-dimensional plane spanned by the distances x and y of the two electrons from the nucleus. The classical motion for this system is fully chaotic and the quantum wavefunctions may be expected to exhibit scars. In fact, a careful implementation of Gutzwiller's semiclassical formula provides excellent predictions for the quantum-mechanical energy levels. The ground-state energy is given to within one per cent and the energy levels for doubly excited states with principal quantum numbers as large as eight or nine are accurate to four significant figures.

The connection of these results with scarred wavefunctions is illustrated in Fig. 3*b*, a topographic map of a numerically calculated wavefunction for an $n = 6$, doubly excited state of helium. This wavefunction is clearly peaked along the path of the classical periodic orbit that is analogous to the right-angle orbit in the Sinai billiard in Fig. 3*a*. In helium this unstable periodic orbit corresponds to an asymmetric stretch of the two electrons. The configuration space in both examples is bounded along the x and y axes (the electrons cannot pass through the nucleus) and to the right by a semicircular potential curve: in the billiard this boundary is a hard wall, in the helium atom it is a smooth barrier determined by the Coulomb attraction of the nucleus.

Because of the similarities, the classical periodic orbits and many of the wavefunctions share common features. But there are also some interesting differences with important physical implications. For example, the doubly excited helium atom is unbounded along the x and y directions, and it is possible for one of the electrons to fall into the nucleus while the other flies off to infinity. In fact, typical chaotic classical trajectories always ionize. Because of this autoionization, the doubly excited states are technically resonances rather than eigenstates, and the quantization of the helium atom is also closely related to the quantum description of chaotic scattering of ballistic electrons in mesoscopic wires with right-angle bends⁸. □

R. V. Jensen is in the Department of Physics, Wesleyan University, Middletown, Connecticut 06457, USA.

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Digital correctness

THE world of extreme hi-fi is riddled with strange postures and pretences. The real aficionado argues the rival merits of amplifiers and loudspeakers in terms of subtleties such as poise, texture and articulation, far beyond mere physical measurement. Fads and fashions abound. For many years valve amplifiers have been held to be purer than solid-state ones; recently even negative feedback has been denounced as too coarse for the truly dedicated; any day now Daedalus expects the original phonograph to be reinstated as the only acoustically correct mode of sound reproduction. And yet, with the coming of digital sound encoding, any sound-reproduction system can be evaluated quite definitively. Daedalus is now doing it.

He simply takes the sound system under test and fits its loudspeaker with a digital position sensor. This generates a stream of binary numbers that encodes its vibration exactly. He then compares this output stream of numbers with the original input stream from the compact disc, digital tape or whatever. This is easily done by feeding both streams simultaneously into a computer. If the sound system is perfect, its output stream of numbers is identical to its input; if not, the deviations show wherein the imperfection lies. Subjective notions of timbre, coherence, coloration and so on, are quite irrelevant.

And this, says Daedalus, is the route to perfect hi-fi. His cunning trick is to feed the input stream to the computer directly, but to delay it slightly on its way to the sound system. The computer is therefore ahead of the sound system, and knows what should be coming next. If the output stream is beginning to deviate from perfection, the computer is in a position to put it right.

In its few milliseconds of running look-ahead time, the computer evaluates the current bit-stream from the speaker. From its mathematical model of the system, it makes an informed guess as to what the next few binary numbers will be. Knowing what they should be, it calculates a corrective 'tweak signal', and feeds it into the amplifier to bring the loudspeaker back on track. The computer's model, derived by continuous evaluation of the sound system's deviations, is steadily updated and optimized. By learning on the job, so to speak, it can work with any sound system at all.

Thus the cheapest amplifier, driving the crudest loudspeaker, can be upgraded to total perfection by one simple accessory. The refined agony of hi-fi comparisons will become a thing of the past. All systems will be perfect, and will sound perfectly alike.

DAVID JONES

Genesis of a pulsar's planets

SIR — Wolszczan and Frail¹ have discovered at least two planets in nearly circular orbits with periods of 66.6 and 98.2 days around PSR1257+12. We propose that these planets formed before the pulsar, originally orbiting a close binary star at a distance of a few AU. The primary star of the binary formed a neutron star. The secondary first acted as an extra mass, to ensure that less than half the system mass was lost in the supernova explosion and the binary and the planets remained bound. The secondary was destroyed in a subsequent phase of mass transfer. The mass flow concomitant with this destruction engulfed the planets, causing their orbits to shrink and circularize. This scheme has the advantage over existing ones that (1) its progenitors are probably quite abundant; (2) it can explain planets around old, recycled pulsars; and (3) the number of planets is not limited to one per pulsar.

Specifically, PSR1257+12 may have begun as a binary of two stars of about $10 M_{\odot}$ with a period of a few days, and a planetary system around its centre of mass in which two planets were at distances 2.5–4 AU. The binary evolved with mass transfer into a massive X-ray binary. Such a system is not disrupted by the explosion that forms the neutron star because the exploding star has a low mass due to prior mass exchange. The velocity change of the binary due to the sudden mass loss and possibly an additional random kick imparted to the neutron star is typically not more than 40 km s^{-1} , ensuring that a planet closer to a $20 M_{\odot}$ binary than 10 AU stays bound.

Next, the neutron star undergoes complete spiral-in into the core of the secondary, forming a so-called Thorne–Żytkow (TZ) object². Such an object will expand and resemble a red supergiant near the end of its life. It will therefore engulf all planets closer than about 3–4 AU, causing their orbits to shrink and become (nearly) circular^{3,4}. For example, for a planet at 3 AU, the spiral-in time, the remaining lifetime of the TZ object and the survival time of the planet in its envelope are similar, 10^3 – 10^4 yr.

Many massive and intermediate-mass ($\sim 10 M_{\odot}$) binaries with planetary systems could have at least one planet suited to our requirements. Also, as massive stars are often born in close binaries, a substantial fraction of pulsars are born in a possible progenitor system. The survival requirement then determines what fraction of pulsars actually retains one or more planets. We predict that pulsars can have both inner planets with orbital radii of about 0.2–3 AU and outer planets with orbital eccentricities increasing with

orbital radius. (The possible third planet¹ is close enough to have been in the TZ object, but its orbit could be less completely circularized.)

The pulsar in our scheme has been embedded almost up to the end of the TZ phase in a dense medium, accreting at the Eddington rate. Therefore, unlike models creating the planets from a slowly emptying disk, it can easily have been spun up to a period of 6 ms. Its low magnetic field ($8.8 \times 10^9 \text{ G}$) may seem unexpected, but we know that field decay can be significant in descendants of massive binaries (for example the binary pulsars 1913+16 and 0655+64). We considered models like the above involving a low-mass X-ray binary, because this is more commonly thought to be the site for forming recycled pulsars (see, however, ref. 5). We believe they can be excluded, because the planets would have to be much closer to the pulsar than is observed to have remained bound after the supernova explosion.

Because PSR 1257+12 is very similar to other disk millisecond pulsars, this may indicate that some fraction of these formed from massive binaries. This has important implications for our understanding of binary evolution and would provide a possible solution to birth-rate problems of millisecond pulsars.

R. A. M. J. WIJERS

Princeton University Observatory,
Peyton Hall, Princeton,
New Jersey 08544, USA

E. P. J. VAN DEN HEUVEL

M. H. VAN KERKWIJK

Astronomical Institute,
University of Amsterdam,
Kruislaan 403, 1098 SJ Amsterdam,
The Netherlands

D. BHATTACHARYA

Raman Research Institute,
Bangalore 560 080, India

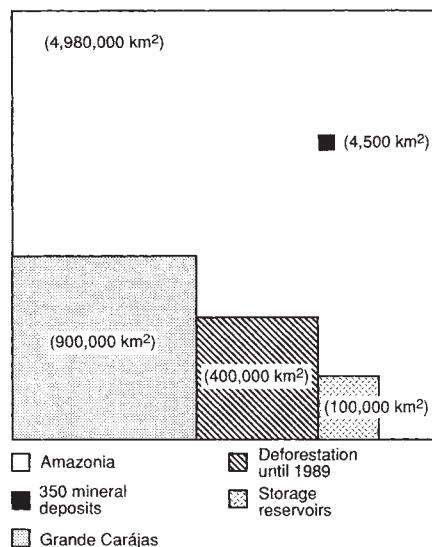
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Mining in the Amazonian rainforests

SIR — Although a sustainable agriculture or timber production is considered possible in some parts of the world¹, such 'sustained' endeavours have failed in the Amazon region. On the other hand, mining in the Amazon is condemned by many environmentalists without any consideration of its positive aspects.

More than 100 million years of Amazonian history in equatorial latitudes, involving crustal bulging during the opening of the South Atlantic and subsequent continental sedimentation without any limestone formation, has resulted in thick laterites and soils poor in nutrients with a very low cation-exchange capacity but high aluminium toxicity². The intense tropical weathering produced depletion of silica and enrichment of certain metals, leading to accentuation of mineral reserves in the Brazilian Amazon^{2–4} (see table).

The prospects for the Amazonian ecosystem appear bleak. The main dangers emanate from large industrial projects, such as *Grande Carajás*; the construction of dozens of hydroelectric



Mining would affect relatively small areas of Brazil's Amazonia compared to other possible uses of the land^{2,5}.

power plants; the erroneous belief that large-scale exploitation of the forests and agronomic resources is a sustainable proposition; and the covert license to thousands of gold-diggers to spoil and pollute the environment with the use of mercury and production of waste dumps, as well as under-use of the resources by recovering less than 50 per cent of the metal. The possibility of any recovery may lie in an understanding of these dangers and/or political intervention such as the recent 'debt-for-nature-swaps' agreement between Brazil and

	Amazon (reserves in 10^6 tons)	Brazil	Global
Fe	18,000	36,000	210,900
Al	2,000	2,500	22,640
Mn	16	56	3,900
Nb	3.2	4.0	4.6
Sn	>0.48	0.63	4.4
Au	0.013	0.033	0.070

the United States. Further, the total maximum area coverage of the exploitable mineral deposits, numbering about 350 (ref. 4), will not exceed 4,500 km² even allowing for a generous estimate of 10 to 15 km² per deposit. A single mining area would also recuperate more rapidly once the deposit is exhausted. But large-scale deforestation may be prevented by such mining only if ore processing is done outside the rainforests (for example in the Brazilian coastal areas, where the required infrastructure already exists), and if access to the mines is strictly controlled to prevent proliferation of settlements.

Brazil has no population pressure, and has vast areas with good soils south of the Amazon. Therefore careful attention should be given to the natural resources of the Amazon and to ways to curtail the destruction of a unique tropical ecosystem and indigenous ethnic groups.

ANDREAS HOPPE

*Geologisches Institut der
Albert-Ludwigs-Universität,
W-7800 Freiburg, Germany*

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Curved saplings at Mount St Helens

SIR — One of the most important effects of the eruption of Mount St Helens on 18 May 1980 was the blowdown of large trees by the lateral blast of hot volcanic ash and gas. Many of the large trees fell in the direction of this flow¹. During a recent visit to the blowdown area, we observed that the flow had a different effect on small saplings, 1–2 m in length. Although Douglas fir and Sitka spruce saplings perished in the high temperatures of the blast, many remained standing even in areas where larger trees had fallen. Another remarkable feature of those saplings that had grown on slopes was that they were consistently curved in the downslope direction, even in regions where the larger trees had fallen in the up- or along-slope direction, parallel to the flow¹. These saplings were also stripped of limbs and bark, and had small cracks on the inner, downslope side of the curve, where they were slightly charred. In contrast, the upslope sides of the saplings were smooth.

Trees typically point downhill at a small angle when growing on a slope, and therefore experience a gravitational torque². Fir and spruce are softwood trees, which grow compressional reac-

tion wood when under mechanical stress. Compression wood has relatively high compressive strength but low tensile strength. The strength of this reaction wood counteracts the net gravitational moment. Experiments suggest that, on drying, compression wood can contract longitudinally a few per cent, which is perhaps ten times as great as the contraction of regular wood³.

We believe that the saplings were curved by differential dehydration of compression wood (downslope) relative to regular wood (upslope) caused by the hot blast. During the eruption, the hot volcanic ash and gas flowed along the ground, felling the larger, more inflexible trees; however, the more elastic saplings remained standing. In several locales east and north of Spirit Lake, we estimated that the radii of curvature of the saplings were 120–240 cm. The average thickness of the saplings was about 5 cm. Based on this geometry, we calculate that the downslope side contracted about 4 per cent more than the upslope side. The presence of small near-surface contraction cracks on the downslope side suggests that slightly more differential contraction occurred following tensile failure of the compression wood. Our estimates of the total differential shrinkage of the saplings are in good agreement with experimental measurements of the shrinkage exhibited by drying both compression and regular wood³.

In contrast to the mature trees which were blown down, saplings cannot be used as flow direction markers. However, the contraction and degree of curvature are functions of the amount of drying. So, sapling curvature, measured directly after an eruption, could be used to infer the temperature and duration of a volcanic flow. It is interesting to note that these saplings are natural analogues of bimetallic strips, except in this case curvature is induced by drying rather than temperature change.

MARCUS I. BURSICK

*Jet Propulsion Laboratory,
MS 183–501, Pasadena,
California 91109, USA*

ANDREW W. WOODS

*Institute of Theoretical Geophysics,
20, Silver Street,
Cambridge, CB3 9EW, UK*

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Scientific Correspondence

SCIENTIFIC Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

Wonky mice and MBP promoter

SIR — Peter Parham¹, in his News and Views article discussing our paper², suggested that the integration sites of transgenes containing the myelin basic protein (MBP) promoter may have influenced the wonky phenotype. Although we agree that such sites may be important in influencing the level of protein expression, we believe that the chances of a transgene integrating into a site involved in normal MBP expression in two independently derived lines are extremely low. In addition, mice with a similar phenotype, resulting from an MBP promoter-driven class I MHC transgene construct, have more recently been reported³.

In many respects, wonky mice are similar to myelinating mutant mice, such as shiverer and *mld*, in which hypomyelination results from a lack of MBP expression. There are also important differences, as wonky mice die by 16–23 days whereas shiverer and *mld* mice survive for 5–6 months⁴. MBP gene expression in wonky mice is greater than in myelinating mutants, with 20–30% of normal MBP RNA levels (data not shown) compared to <1% in shiverer mice⁵ and 3% in *mld*⁶. The myelin in shiverer and *mld* mice, where present, is poorly compacted and the major dense line is absent⁷; in wonky mice, the limited amount of myelin present is thin, yet compact and contains the major dense line. The fact that wonky mice and all the myelinating mutants, including Jimmy (in which the defect is in the proteolipid protein gene, not the MBP gene)⁸, have a similar behavioural phenotype at around 12 days of age can be explained by the hypomyelination which becomes obvious at this stage of development even though the cause differs in each case.

We do not discount the role of the immune system in the pathogenesis of multiple sclerosis. We are simply proposing that increased levels of MHC molecules may *per se* be associated with dysmyelination.

A. M. TURNLEY

G. MORAHAN

P. BARTLETT

J. F. A. P. MILLER

*Walter and Eliza Hall Institute of
Medical Research,
Royal Melbourne Hospital,
Victoria 3050, Australia*

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Rodent polyphyly?

SIR — From phylogenetic analyses of amino-acid sequence data of several proteins, Graur *et al.*¹ suggested that the order Rodentia may not be monophyletic, and that the guinea-pig-like rodents (Caviomorpha) may have a separate origin within mammalian evolution from that of the rat-like rodents (Myomorpha) and the squirrel-like rodents (Sciuromorpha). This suggestion radically contradicts the traditional view of rodent monophyly, based most on comparative morphology. Graur *et al.* used a maximum parsimony (MP) method, but it is known that this method is sometimes misleading, particularly when the evolutionary rate differs among lineages^{2,3}. Therefore, we have re-examined the data by the maximum likelihood (ML) method^{4,5}, which is robust against the violation of rate constancy³.

The sequence data used in our analysis are from SWISSPROT data library, and are mostly the same as those used by Graur *et al.* We additionally used factor IX sequences⁶ with factor X as an out-

group. Three different models for amino-acid substitutions were used in our ML analysis, and the results were compared with those of the MP analysis by the PROTPARS program of Felsenstein's PHYLIP package also used by Graur *et al.* The table shows the results of our analysis on the relationship among Caviomorpha, Myomorpha and Primates. Although the MP method strongly favours the rodent polyphyly tree III: ((Myomorpha, Primates), Caviomorpha) with the bootstrap probability of 0.96 over the traditional tree I: ((Caviomorpha, Myomorpha), Primates) and another rodent polyphyly tree II: ((Caviomorpha, Primates), Myomorpha) consistently with Graur *et al.*, the ML method does not give any significant preference of tree III irrespective of the assumed model for amino-acid substitutions and does not discriminate between trees I and III. When Artiodactyla data are added (lipocortin I is excluded because only a short segment has been sequenced for cow), the MP method gives a bootstrap probability of as high as 0.92 for a rodent polyphyly tree:

(Caviomorpha (Myomorpha (Primates, Artiodactyla))) which Graur *et al.* preferred among 15 possible trees. On the other hand, the ML methods based on the Dayhoff and Proportional models give low probabilities of 0.20 and 0.18 for the tree, and give subtotals of bootstrap probabilities of 0.69 and 0.76 for the rodent monophyly trees.

From these results, we conclude that Graur *et al.*'s claim of rodent polyphyly is premature from the data accumulated up to now, and that their suggestion may represent an example of the fact that unequal evolutionary rates can mislead MP analysis^{2,3}. Nevertheless, we admit the possibility that the phylogenetic distance between Caviomorpha and Myomorpha is large enough to warrant a separate ordinal status for the Caviomorpha because of the difficulty in discriminating between trees I and III.

MASAMI HASEGAWA

YING CAO

JUN ADACHI

*Institute of Statistical Mathematics,
4-6-7 Minami-Azabu, Minato-ku,
Tokyo 106, Japan*

TAKA-AKI YANO

*College of Arts and Sciences,
Showa University,
Fuji-Yoshida, Yamanashi 403, Japan*

ML AND MP ANALYSES OF AMINO-ACID SEQUENCES OF PROTEINS												
Tree	Crys	Lact	Hb α	Hb β	NGF	Fac9	Ribo	Insu	Lipa	Cort	Total	Pr
ML method with Dayhoff model												
I	ML	ML	-5.6 ±7.9	-0.5 ±5.0	ML	-4.0 ±3.5	-3.5 ±3.0	-1.6 ±2.3	-3.1 ±3.5	-8.2 ±6.9	-4.7 ±16.0	.3608
II	-0.4 ±7.0	-4.2 ±5.3	-10.5 ±6.0	-3.3 ±3.6	-9.1 ±6.0	ML	ML	ML	ML	-10.5 ±6.1	-16.1 ±14.0	.0515
III	-5.6 ±4.5	-5.5 ±4.5	ML	ML	-2.2 ±8.3	-3.8 ±3.5	-3.0 ±3.5	-1.6 ±2.3	-0.2 ±4.7	ML	ML	.5877
ML method with Proportional model												
I	ML	ML	-6.6 ±10.2	-1.9 ±5.4	ML	-2.9 ±4.0	-3.8 ±3.9	ML	-3.8 ±6.2	-8.8 ±7.9	ML	.5441
II	-6.3 ±9.1	-5.8 ±4.7	-13.4 ±7.8	-4.3 ±4.1	-12.1 ±7.1	ML	ML	-1.7 ±2.5	-0.7 ±7.3	-10.5 ±7.3	-26.9 ±16.8	.0118
III	-11.0 ±7.6	-5.7 ±4.6	ML	ML	-5.6 ±9.1	-4.0 ±3.5	-2.9 ±4.5	-1.2 ±2.8	ML	ML	-2.5 ±20.2	.4441
ML method with Poisson model												
I	ML	ML	-3.3 ±9.4	-2.8 ±5.5	ML	-3.2 ±4.1	-3.2 ±3.5	ML	-4.9 ±6.6	-8.5 ±8.5	ML	.5598
II	-5.9 ±9.5	-6.3 ±5.1	-10.9 ±6.5	-4.8 ±4.4	-11.6 ±7.1	ML	ML	-1.9 ±2.7	-1.5 ±7.8	-10.6 ±7.7	-27.5 ±17.5	.0110
III	-11.2 ±7.7	-5.9 ±5.2	ML	ML	-5.0 ±9.1	-4.3 ±3.6	-2.0 ±4.3	-1.5 ±2.9	ML	ML	-4.0 ±20.4	.4292
MP method												
I	MP	5	4	1	1	1	3	1	9	4	17	.0303
II	2	MP	8	5	5	MP	MP	1	6	5	20	.0108
III	3	4	MP	MP	MP	3	2	MP	MP	MP	MP	.9589

Tree 1 ((Caviomorpha, Myomorpha), Primates); tree II ((Caviomorpha, Primates), Myomorpha); tree III, ((Myomorpha, Primates), Caviomorpha). ML analyses were performed by three models for amino-acid substitutions; the Dayhoff model⁵ assumes an empirical transition matrix, compiled by Dayhoff *et al.*⁷, and the Proportional and Poisson models assume that the probability of an amino-acid *i* being replaced by another amino acid *j* during an infinitesimally short time interval of *dt* is given by $u d\tau_{ij}$ (τ_{ij} is the frequency of *j*, and *u* is a parameter that determines the substitution rate)⁴ and by $u d\tau$, respectively. For the ML analyses, the highest likelihood tree for each protein is indicated as 'ML', and the differences of log-likelihoods of alternative trees from that of the ML tree are shown with their s.e. following $\pm$ estimated by Kishino and M. H.'s formulae⁸. 'Total' refers to summation of the log-likelihood differences from the diverse proteins. For the MP analyses, the most parsimonious tree is indicated as 'MP', and the differences of numbers of substitutions of alternative trees from that of the MP tree are shown. *Pr*, bootstrap probability for being the ML or MP tree among alternatives during bootstrap resampling estimated by the RELL (resampling of estimated log-likelihoods of respective sites or, for the MP method, resampling of estimated numbers of substitutions) method given in ref. 5. (sample size of 10⁴). Investigated proteins: Crys, α -crystallin A; Lact, α -lactalbumin; Hb α , α -globin; Hb β , β -globin; NGF, β -nerve growth factor; Fac9, factor IX; Ribo, pancreatic ribonuclease; Insu, proinsulin; Lipa, lipoprotein lipase; Cort, lipocortin I.

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Biased DNA repair

SIR — Hennecke *et al.*¹ report the first *in vitro* characterization of a DNA mismatch endonuclease. This enzyme, the *vsr* gene product, initiates very short patch repair of *E. coli* by nicking one strand of the duplex next to the T at a mismatched T:G in the sequence CTWG or TWGG. This specificity confirms extensive *in vivo* data on the *vsr*-initiated system². Sequence-specific nicking is the first step in the repair of certain T:G mismatches to C:G and is apparently designed to target the mutation hot spot caused by 5-methylcytosine deamination to thymine at the *dcm* site: 5' C T W G G 3', where the underlined T is mismatched with G; and 3' G G W ^mC C 5', where W is A or T and ^mC is 5-methylcytosine.

However, methylation on the unmethylated strand, C^mWGG, in the *dcm* site is not required for the function of the enzyme¹. There is an interesting con-

STATISTICAL ANALYSIS OF TETRANUCLEOTIDE SEQUENCES

Tetranucleotide	Rank*	Representation†		Residual‡
		Observed	Expected	
CTAG [§]	256	0.04	< 0.08	-5
TAGG	254	0.10	< 0.11	0
CCTA	252	0.12	< 0.15	-1
CTTG	230	0.21	< 0.26	-1
CAAG	221	0.23	< 0.36	-6
CCAA	195	0.27	< 0.36	-4
TTGG	194	0.27	< 0.44	-7
CAGG	54	0.51	> 0.41	+4
CCAG	50	0.51	> 0.42	+3
CCTG	29	0.57	> 0.53	+1
CTGG	1	0.97	> 0.83	+4

* Rank is in order of abundance⁷, that is, CTAG is the rarest and CTGG the most common tetranucleotide in the *E. coli* genome.

† Representation is based on second-order Markov analysis, which eliminates the effect of deviations in trinucleotide frequencies.

‡ The residual is a measure of the probability of the observed versus expected ratio. For a random sample, the probability of a residual greater or less than 3 is 10^{-3} (ref. 7).

§ The underlined T represents the *vsr* target when T is mismatched with G.

sequence of this methyl-independent feature of the repair system that explains the extreme rarity of certain tetranucleotides in enterobacteria. Although postreplicative deaminations would be correctly repaired by *vsr*, the erroneous incorporation of G across from T in this sequence context during replication will be mistakenly repaired from T to C by the *vsr*-initiated system on those occasions when *vsr* repair occurred before daughter strand repair³. The same result would occur for a G:T mismatch detected during recombination. This preferential T-to-C repair by *vsr* would result in selection against the sequences CTAG, CTTG, TAGG and TTGG and their complements CAAG, CCTA and CCAA. We were prompted to test this possibility because CTAG was already known to be exceptionally rare in the *Escherichia coli* and other enterobacterial genomes⁴⁻⁹, although no adequate explanation was available. Also, *vsr* homologues had been detected by Southern analysis in several enterobacteria including *Shigella*, *Salmonella*, *Klebsiella* and *Citrobacter* (M. Lieb and A. Bhagwat, unpublished data).

We used a statistical analysis of tetranucleotide frequencies^{6,7} from a large sample of *E. coli* genomic DNA sequence (kindly provided by K. Rudd, NLM, Bethesda). The position of these tetranucleotides are listed in the table in the rank order of all 256 tetranucleotides. All are among the rarest 25% of tetranucleotides and three are among the five rarest tetranucleotides. The expected frequencies of these tetranucleotides were calculated by second-order Markov chain analysis^{4,6,7,10}, which takes into account the effect of trinucleotide frequency. All the potential *vsr* targets are underrepresented by this measure, and CTAG, CAAG, TTGG and CCAA are among the most significantly underrepresented tetranucleotides in the

genome. A few of the other tetranucleotides are also rare, such as GGCC⁷. Perhaps there are similar phenomena acting on such sequences.

Our hypothesis predicts that the transition products of inappropriate *vsr*-initiated repair, CTGG, CCTG, CCAG and CAGG, would be more common than might otherwise be expected. The table shows these four tetranucleotides are among the most common 25% and three are among the most significantly over-represented, after controlling for trinucleotide frequency by Markov chain analysis. The tetranucleotides listed in the table are not unusually rare or common in samples of *Saccharomyces cerevisiae*¹¹ or *Bacillus* genomes (data not shown). These species have not been shown to have a *vsr* system with the specificity found in *E. coli*.

The function of *dcm* methylation remains a mystery, but the repair system that accompanies it may have had a profound effect on the whole genome. Selection against the sequences recognized by *vsr* can explain the extreme rarity of certain tetranucleotides in enterobacterial genomes. These tetranucleotides may turn out to be hot spots for spontaneous mutations. This bias in repair should be enhanced in *dam*- or *vsr* overexpressing mutants.

M. McCLELLAND

California Institute of Biological Research,
11099 North Torrey Pines Road,
La Jolla, California 92037, USA

A. S. BHAGWAT

Department of Chemistry,
Wayne State University,
Detroit, Michigan 48202, USA

FRTZ ET AL. REPLY — By means of an argument based on our recent description of the *vsr* DNA mismatch endonuclease¹ and a statistical analysis of available *E. coli* sequence data,

McClelland and Bhagwat describe a plausible mechanism by which certain tetranucleotide sequences are depleted from the *E. coli* K-12 genome. In essence, this mechanism rests on competition of two different DNA mismatch repair pathways for processing with opposite strand bias the same base/base mismatch, a G residue misincorporated during replication opposite T in a special sequence context. The existence of such competition *in vivo* was, indeed, demonstrated earlier¹².

We have independently carried out a statistical analysis. The data set was taken from the latest release of the ECD *E. coli* database^{13,14} and comprised all contiguous stretches of DNA sequence longer than 5,000 nucleotides in a strand-specific fashion (reading clockwise 5' to 3' on the *E. coli* map). Second-order Markov chain analysis⁶ was used to calculate expected frequencies of occurrence from trinucleotide frequencies as input. Phillips *et al.*⁶ have already pointed out that this procedure eliminates carry-over of distortions of trinucleotide frequencies. Our results are very similar to those obtained by McClelland and Bhagwat. We agree in the conclusion that these data lend strong support to the model sketched above. Our results, together with the mechanistic explanation of over- and under-representation of certain tetranucleotide sequences — essentially the same model as put forward by McClelland and Bhagwat — were presented by one of us (M.K.) at the Banbury Conference "The *E. coli* Genome-Sequence Acquisition and Interpretation" in October 1991.

HANS-JOACHIM FRITZ

RAINER MERKL

Institut für Molekulare Genetik,
Georg-August-Universität Göttingen,
W-3400 Göttingen, Germany

MANFRED KRÖGER

Institut für Mikrobiologie und
Molekularbiologie,
Justus-Liebig-Universität Giessen,
W-6300 Giessen, Germany

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Old myths and new

Ron Naylor

In the Wake of Galileo. By Michael Segre. *Rutgers University Press*. 1991. Pp. 192. \$27.95.

THE age-old myth of Galileo as the inveterate experimenter who dropped spheres, has become part of European folk belief. The far less well known, more arcane and somewhat titillating myth long favoured by many scholars is that he hardly used experiment at all — and thought very little of it. The recognition that myths arise in narratives created after the event by others ought, one would expect, to direct us towards the words and actions of Galileo in order to solve the enigma of his experimentation. Strangely, Michael Segre recommends the very opposite — that we seek instead the answer in the works of Galileo's contemporaries and disciples.

Over the past two decades, dramatic developments in the interpretation of Galileo's work have utterly transformed the estimation of his achievement for many — but not it seems for Segre. It now looks as if Galileo was a far more versatile and sophisticated experimenter than anyone had dreamt. The careful study of his surviving, unpublished experimental notes, preserved in Florence and long regarded as of little importance, has permitted the reconstruction of a series of previously unknown experiments, experiments never referred to in his published work or described in his correspondence. (It has proved possible to reproduce the data recorded cryptically in the manuscripts.) The discovery stunned and roused from their dogmatic slumber a generation of historians who had been weaned on the view that Galileo had little real use for experiment. They had been strongly influenced by the rationalist intellectualism of the eminent historian Alexander Koyré who treated both 'materialist' and 'empiricist' interpretations of science and history with equal disdain. He poured scorn on the popular view that Galileo relied heavily on experiment, particularly to develop his revolutionary theory of motion.

Despite this change of heart, Segre has decided that it is possible to write a book on Galileo's work and influence without any discussion of the new evidence, claiming that the whole matter is too complex for detailed consideration. It is as if Segre had decided to leave the issue of Galileo's known experimentation to one side. The reality of the situation, though, is that crucial lessons and valuable insights can be had from the manuscripts, even if some of the

experiments are not straightforward. For we now know that not only did Galileo spend time and care in experimentation but also that this experimentation underpinned his research technique. Segre mentions none of this.

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Galileo — unpublished manuscripts vindicate his position as inveterate experimenter.

In his final and greatest work, the *Discorsi e Dimostrazioni Matematiche, intorno a due nuove Scienze* of 1638, completed at the end of his life while under inquisitorial house-arrest, Galileo was preoccupied with the need to present his theoretical ideas as simply, clearly and convincingly as possible. The very notion of mathematically formulated experiment was revolutionary, although he could not risk the inclusion

of complex experiments, particularly those that had produced some anomalous results. So in being compelled to tidy up his evidence, he had to omit large chunks of it entirely.

In the *Discorsi*, Galileo emphasizes the value and importance of experiments yet provides accounts of relatively few — the inclined plane experiment being an outstanding and well-known example of an occasion when he did. This seemingly contradictory behaviour allowed Koyré to argue that Galileo's experiments were no more than thought experiments used to persuade readers. Furthermore,

Mary Evans

Koyré was convinced that Galileo's claims of accuracy were so good as to be almost unbelievable. In fact, he was convinced that Galileo had described experiments that were physically impossible.

While apparently maintaining his allegiance to Koyré, Segre totally repudiates Koyré's claim to have been able to establish Galileo's method from the study of Galileo's work (probably because Koyré's original claims are now widely held to be not only mistaken but also essentially rhetorical). Nevertheless, Segre re-poses all the old questions once posed — and answered — by Koyré and his followers: "What role did Galileo himself assign to experiment? Did he use it as a basis for theory construction? As a final test? Or only as a didactic demonstration? These questions cannot be answered on the basis of either his work or his manuscripts." Coming out of the blue, this newly found scepticism leaves one with the impression that the most successful explanations of

Galileo's work and manuscripts are simply unacceptable to Segre.

All is not lost, however, for Segre claims that the answer to all the questions posed is to be found in the work of Galileo's contemporaries and disciples. But although Segre's discussion of the work of Galileo's followers, particularly Evangelista Torricelli and Bonaventura Cavalieri, provides some interesting perspectives, it does not really take his

main thesis any further. The insights gained are certainly no easier to interpret than Galileo's work and can be of value only when seen in relation to this work.

But the overriding contradiction in Segre's view is this: if Galileo's copious writings, notes and correspondence cannot yield reliable evidence, why should we expect the work of his critics or disciples to do so? Where in fact would the corrosive effect of such scepticism end?

Segre concludes that "throughout we have seen that it was Galileo's critics who employed an empirical discourse", from which he deduces that it was they, rather than Galileo, who were the real empiricists. Alone, however, this argument can prove very little — one needs to be rather cautious about the idea of 'empirical discourse' and enquire a little into the motivation of those employing it. Several of Galileo's contemporaries were so struck by his proposal of mathematical-empirical laws that they became obsessed with his idea of empirical verification. But rather than roll smooth spheres down a gently inclined plane as Galileo had done, they decided to study the vertical fall of spheres from high towers. Naturally, because they did not conduct their experiments under the carefully controlled conditions recommended and used by Galileo (as the manuscripts now confirm), they insisted his law of fall was not quite correct and proposed some 'empirical' improvements to it. Others challenged the accuracy of his claim that the pendulum was isochronous. All of them implied that they were the better 'empiricist'.

So Galileo's critics tended to become bogged down in empirical trivia, mainly because their use of experiment did not form part of a directed research programme. Indeed, they wrote a great deal about their empiricism — in relation to Galileo's ideas it should be noted — because that was all they were capable of doing. They failed to see how empiricism could be used to develop physical theory. Certainly they did find some flaws in Galileo's empirical claims, but his theory of motion was so sound overall that these criticisms had little effect.

That Galileo's critics had a notably inferior grasp of the use of experiment, yet stressed how 'empirical' they themselves had been, is no basis for dismissing Galileo's actions and words. In reality, the very inability of his rivals to distinguish the wheat from the chaff demonstrates his superior empirical abilities in comparison. □

Ron Naylor is in the Department of Philosophy, Thames Polytechnic, Wellington Street, Woolwich, London SE18 6PF, UK.

Before or after?

Lynn Bindman

Long-Term Potentiation: A Debate of Current Issues. Edited by Michel Baudry and Joel L. Davis. MIT Press: 1991. Pp. 454. £62, \$70.

A PRIME candidate for the neuronal basis of learning and memory is a phenomenon called long-term potentiation. This is an increase in the strength of synaptic transmission following increased activity at a synapse that lasts for hours and possibly weeks. It has been produced in the hippocampus, cerebral cortex and cerebellum, all areas of the brain that are important for learning and memory.

The explosion of interest in long-term potentiation (LTP) is illustrated by the fact that four sessions of the Society for Neuroscience meeting in November 1991 were devoted to the phenomenon. I feared that this book, arising from a conference held in 1990, would seem outdated and be merely another collection of symposium papers. Instead, I found it to be compelling reading and a most worthwhile compilation of information and references (updated into 1991). This is due as much to the way in which the editors have organized the book as a debate of five controversial aspects of LTP, ensuring that each author addresses the debated issue and arranging a discussion chapter at the end of each section, as to their choice of distinguished contributors. In most places, the excitement of the debate and current research shines through.

The five themes of the book are: first, whether the maintenance of LTP depends on presynaptic or postsynaptic mechanisms; second, the role of different calcium-dependent enzymes; third, the biophysical and structural changes that underlie different stages of LTP; fourth, the relation between LTP and learning and memory; and fifth, the rules for LTP in neural networks. The book therefore encompasses experimental approaches that range from the cellular to the behavioural.

The presynaptic versus postsynaptic question is the dominant one in the debate, and so it is appropriate that both here and in the book it receives the most attention. Evidence from more than one laboratory indicates that the maintenance of LTP in the hour or so after induction results from an increase in neurotransmitter (glutamate) release. Because there are two main classes of glutamate receptor, the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and the NMDA (*N*-methyl-D-aspartate) classes, increased glutamate

release should enhance both the AMPA- and the NMDA-mediated components of the postsynaptic potential. But in 1988, J. Kauer and associates, and D. Muller and G. Lynch found that the enhancement of excitatory postsynaptic potentials in LTP was mediated by only the AMPA class. In the book, though, Z. Bashir and colleagues, and R. Malinow and R. Tsien present convincing evidence that the NMDA receptor-mediated component is also enhanced in LTP. It is still unclear why these contradictory findings exist, although Malinow and Tsien have tested various possibilities and proffer a still untested explanation.

Quantal analysis of transmitter release is another tool for differentiating between presynaptic and postsynaptic maintenance of LTP. Three groups assess different methods of quantal analysis at synapses in the central nervous system. The interpretation of the results of these analyses is controversial because such synapses have different characteristics from the neuromuscular junction where the technique was first applied. The results using quantal analysis of transmitter release in LTP are also not in agreement, favouring either a primarily presynaptic or a mainly postsynaptic change.

It is obvious from the work reported in the book and at the Society for Neuroscience meeting that both presynaptic and postsynaptic changes can occur in LTP. The debate must now focus on the circumstances under which these changes are produced, and the time over which they are expressed.

What are the new findings and issues that are missing from the book? Since it was written, associative, synapse-specific LTP has been induced in single cells of the motor cortex of awake cats; nitric oxide has emerged as a candidate for the retrograde messenger in the induction of LTP; a form of LTP not involving NMDA receptors has been induced in area CA1 of the hippocampus; microfluorimetry has shown increased concentrations of calcium within dendritic spines after afferent tetani; and the mechanisms underlying the obverse phenomenon of long-term depression are being identified. But these omissions — inevitable when book publication takes many months, and most obvious in a fast moving field such as LTP — should not deter libraries and laboratories from purchasing this excellent book. □

Lynn Bindman is in the Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK.

■ *Excitatory Amino Acids and Synaptic Transmission* edited by H. Wheal and A. Thomson is a comprehensive reference text that includes a section on LTP and an extensive glossary. Published by Academic, £65.

Water under the bridge

William H. McNeill

The Geography of Science. By Harold Dorn. *The Johns Hopkins University Press*: 1991. Pp. 219. \$41.50, £26.

ACADEMIC banter, Dorn explains, led him to undertake a course on "science in the Asiatic mode of production" — an enterprise that eventually produced this slim volume on the role that geographical setting plays in the development of scientific thought. The book is not well titled, because the rich variability of geographical environments is conspicuously absent from its pages. Instead Dorn proposes a simple — to my mind an unduly simple — bipolar pattern for the development of science, pitting "Oriental science", characterized by bureaucratic organization and practical value, against "Hellenic science", characterized by individual activity without any practical application.

Dorn argues that what he calls "Oriental science" reflects the importance of irrigation and other forms of water engineering. In oriental societies, governments needed scientists for practical tasks, especially calendrical calculations, and thus organized scientists bureaucratically. In turn, this "hydraulic hypothesis", as he calls it, "belies the notion of a 'unitary science' in the ancient world. Instead science would appear to have a double root — Oriental and Greek".

After setting up this dichotomy, Dorn examines four instances where he believes that the bureaucratization of science was influenced by demands for water engineering. He chooses Ceylonese, Chinese, Mayan and Pueblo Indian examples, presumably because the diversity of these cultures means that any convergences of their science may be interpreted as reflecting the importance of water engineering in shaping their science. What he finds, of course, is organized concern about the calendar, although the evidence of 'bureaucratic science' among the Pueblo Indians is slender to say the least, limited to markings on a cave wall that probably recorded the solstices accurately with the help of holes in specially constructed sighting stones.

Dorn then devotes a chapter to ancient Greek science, and proceeds to analyse how the antithetical traditions of Greek and Oriental science mingled in the Hellenistic, Roman, Moslem and Byzantine worlds. This is followed by a chapter on science in Europe above the 40th parallel, in which he concentrates almost wholly on the so-called 'scientific revolution' of early modern Europe. There, he declares, a new basis for state power, based largely on gunpowder

weaponry, created a new kind of official need for practical science without, however, extinguishing the Hellenic heritage of useless, individual scientific research. Dorn finishes by showing how water engineering in what was once called the Great American Desert now provides the principal basis for the bureaucratization of science in the United States.

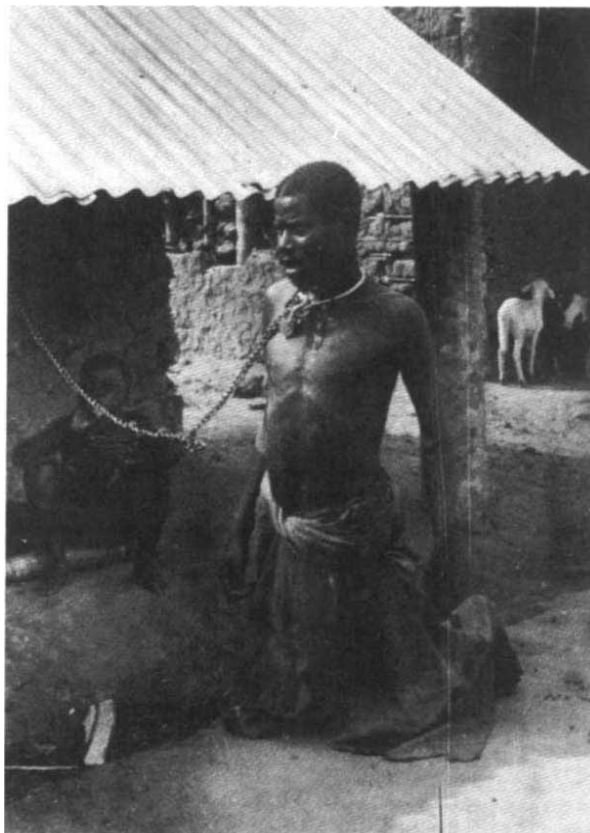
Dubious assertions abound in Dorn's pages. I doubt, for example, whether Philip of Macedon is properly dubbed a "water king" or that his consolidation of power depended on water engineering. Similarly, the remark that "technology in contrast with science, was historically an affair of the country rather than the town" seems so lopsided as to be plainly wrong. He cites mining and the construction of aqueducts and canals as evidence for the rural character of technology; but what about all the artisanal skills that centred in towns? Out-and-out errors also suggest that Dorn's effort to explore the Asiatic mode of production across space and time led him to investigate unfamiliar materials too hastily. He tells

us, for instance, that the Near East "was intensely hydraulicized but failed to achieve an Iron Age" and, more trivially, that Attalus of Pergamum set up his famous library "upon his inheritance of Persia." Historians will be surprised by both assertions.

Dorn does offer a few shrewd observations. In arguing that applied science in Europe "is largely a nineteenth century development", he points out that "the niche that applied science currently occupies in industrial societies was already filled in early modern Europe by the occult sciences — alchemy, astrology and magic."

This observation strikes me as persuasive, but it calls into question the simple dichotomy around which Dorn builds his book. His account of Greek science — pure and individual, and without any institutional base whatsoever — can be maintained only by banishing pythagorean and other occult or semi-occult forms of speculation from the purview of 'science'. But such a separation is profoundly unhistorical. Mystical and some quite material theories, for example the humours of galenic medicine, had very practical ends in view and played a prominent part in Hellenic and all subsequent science. And, as the history of freudianism shows, this form of science remains alive and kicking today.

So the contrast between the two types of science that Dorn proposes is to my



In 1901, a devastating epidemic of human trypanosomiasis, or sleeping sickness, erupted in Uganda, killing more than 250,000 people. The Europeans had seen their colonization of Africa as a civilizing mission, bringing with it the gift of Western medicine. But by the 1960s and independence, many Africans had come to regard sleeping sickness as the 'colonial disease', mainly because of the truly draconian measures that were taken by some colonial administrators to check its spread. Pictured here is one of a series of lazarets that made up the *cordon sanitaire* around the Uele district in the Belgian Congo. The social history of the disease in northern Zaire between 1900 to 1940 is the subject of *The Colonial Disease* by Maryinez Lyons. Published by Cambridge University Press, price £50, \$79.95.

mind quite unconvincing and does almost as much injustice to what he calls Oriental as to Hellenic thought. The fact is that abstract and entirely impractical speculation was not absent from the ancient Mesopotamian mathematics, or from Indian and Chinese science, any more than Hellenic 'science' was 'pure' and impractical. Dorn's hydraulic hypothesis seems archaic, having been almost universally repudiated since Karl Wittfogel first advanced it 35 years ago. Similarly, his view of science as either pure or applied, true or false, Hellenic or Oriental harks is back to a positivist vision of the history of science that George Sarton transplanted to the United States 75 years ago. Today's historians are almost unanimous in their rejection of this vision, which mistakenly identifies science with some accepted truths of our time, arbitrarily abstracted from the thoughts of civilizations past. □

William H. McNeill is Professor of History emeritus at the University of Chicago, 36 Schoolhouse Road, PO Box 45, Colebrook, Connecticut 06021, USA.

Raiders of the last ammonite

Henry Gee

On Methuselah's Trail: Living Fossils and the Great Extinctions. By Peter Douglas Ward. W. H. Freeman: 1991. Pp.214. \$18.95, £14.95.

HERE is a great idea for a movie. It would be a pacy, all-action adventure, in which the hero overcomes impossible odds to achieve some arcane and rather dotty objective. I favour a palaeontological theme — fossils are guaranteed box-office draws and attract the right kind of teenage audience. No swash would be left unbuckled as our gritty and almost certainly stubbly hero (Harrison Ford, perhaps?) strives singlemindedly to wrest the last ammonite from crumbling Upper Cretaceous cliffs before his rope gives way or (worse) he gets cut off by the tide.

I have found just the script, a short book called *On Methuselah's Trail* by a real-life palaeontologist. Do not be fooled by Ward's intention, which is to discuss why some animals thrive and survive for millions of years virtually unchanged, seemingly oblivious to the tide of evolution flowing all about them. Ward allows us rare glimpses into the biology of some of his favourite 'living fossils', but what *Methuselah* is really about is adventure. Ward takes us deep-sea diving in Puget Sound in pursuit of brachiopods, and tells us what it is like

Coping with the cold

Andrew Cossins

Animal Life at Low Temperatures. By John Davenport. Chapman and Hall. 1991. Pp. 246. £29.95.

COLD is a familiar enough sensation for us warm-blooded creatures, yet our appreciation of its profound biological significance tends to become blunted by domestic central heating. This new book provides a highly readable account of the ways in which animals of all types cope with a cold environment. It is an introductory text aimed squarely at advanced undergraduates looking for a broad but not over-detailed account and perhaps specialists who need to familiarize themselves with some basic environmental physiology.

Because cold adaptation encompasses most of thermal biology, much of the book's contents correspond rather close-

ly to surface just in time to avoid the 'bends'. He recounts a thrilling race against time to prepare *Nautilus* specimens for export in an ill-equipped laboratory on a Pacific island (no other dissection guide is so compulsive). He takes us motoring to ruined castles in the French Pyrenees, geological hammer in one hand, Michelin guide in the other, death-defying French drivers on the bumper. He takes us fossil-collecting among the grim villages of the Basque country, where you can turn from the rocks to find a terrorist pointing a rifle up your nose. To subvert what Tom Lehrer once said about the obituary of a Viennese socialite, *Methuselah* is the juiciest, spiciest, raciest palaeontology text I have ever read: once one has overcome the appallingly patchy way in which it has been put together (more a collection of writings than a coherent book) it is as unputdownable as any airport-lounge thriller.

What strikes one rather forcibly is Ward's strongly visual evocation of palaeontology in general and fossil-collecting in particular. Each fossil locality has its own character, associations and mythology. And what with the exciting interludes set under water or on tropical islands, each chapter reads as though it were made for television, a sort of horny-handed *Life on Earth*. The thought of that venerable television series, staple of zoology courses everywhere, makes me think that my movie might be a bit too preppy for Harrison Ford. Kevin Costner, anyone? □

Henry Gee is an assistant editor of *Nature*.

ly with other popular texts in comparative and environmental physiology. Davenport admits to being an ecophysiologicalist with strong behavioural leanings, and what distinguishes the book is the coverage of behavioural responses, such as huddling and shelter, and sections devoted to humans in a cold environment and to cold and evolution. This will make it a good source for the more ecologically minded reader and a useful resource for teaching.

The other distinguishing feature of the book is its sheer readability, with concepts, relationships and critical analysis being presented in a very accessible form. I particularly liked Davenport's clear statements of where he stands on certain controversial issues such as the evolution of endothermy, the evidence relating to endothermic dinosaurs and the existence of metabolic cold adaptation in polar fish species.

On the debit side there is a problem of balance. Cold presents different problems for animals depending on whether they are endothermic homeotherms or poikilotherms. For endothermic homeotherms, the problem is how to increase thermogenic heat production in a food-scarce environment. Poikilotherms, on the other hand, must overcome or prevent freezing of their tissues. Both of these aspects are well covered in what amounts to roughly half of the book.

But many poikilotherms also have large problems in dealing with cold at temperatures well above zero, and on this subject Davenport has virtually nothing to say. There is no mention of chill injury, chill coma, resistance acclimatization or its underlying physiology. These are widespread and important phenomena and their absence is a serious omission in a teaching text of this sort. That is a shame, particularly as the author includes some really basic biology, such as the structure of the insect respiratory system and of biological membranes, knowledge that could be taken for granted in the advanced undergraduates who would profit most from the book. □

Andrew Cossins is in the Department of Environmental and Evolutionary Biology, University of Liverpool, Derby Building, PO Box 147, Liverpool L69 3BX, UK.

New in paperback

■ *Lonely Hearts of the Cosmos: The Story of the Scientific Quest for the Secret of the Universe* by Dennis Overbye. For a review see *Nature* **351**, 534; 1991. Published by HarperPerennial, price \$13.

■ Volumes 1 and 2 of *Perspectives of non-linear Dynamics* by E. Atlee Jackson provide a broad introduction to this field. The book is aimed at students, teachers and researchers alike. Published by Cambridge University Press, each at £19.95, \$32.95.

Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*

Richard Losick & Patrick Stragier

Sporulation in *Bacillus subtilis* is a model for how cells of one type generate other differentiated cell types. During sporulation two cellular compartments arise that differ from each other and from the progenitor cell. Differential gene expression between the two is governed by the successive appearance of four transcription factors whose activities are coordinated in crisscross fashion between the two cells.

How do cells of one type give rise to dissimilar types of cells? Much has been learned from the study of simple differentiating microbial systems, including the interconversion of **a** and **σ** mating cell types of the budding yeast *Saccharomyces cerevisiae*¹, the formation of swarmer and stalked cell types by the dimorphic bacterium *Caulobacter crescentus*² and the formation of heterocysts by the cyanobacterium *Anabaena*³. We now describe a particularly powerful microbial system for the study of differentiation—the formation of two distinct cell types by the spore-forming bacterium *B. subtilis*^{4,5}. The expression of different genes in the two cell types is chiefly governed by four transcription factors. The action of these factors is restricted to one or the other cell and is coordinated by communication between the two cells.

In sporulation, an asymmetrically positioned septum partitions the developing cell (the sporangium) into two unequal compartments, the forespore and the mother-cell, each of which carries a chromosome from the last round of vegetative DNA replication (Fig. 1 *a, b*). The two compartments follow different programmes of gene expression: certain sets of genes are transcribed in the forespore and others in the mother-cell. After this initial stage, the septum migrates around the forespore (Fig. 1*c*), eventually engulfing it, pinching it off as a free protoplast (Fig. 1*d*) wholly enclosed within the mother-cell.

In the final stages of differentiation, a cortex of cell wall material is produced in the space between the mother-cell and the forespore membranes and coat polypeptides from within the mother cell are deposited around the forespore to form the tough protein shell encasing the mature spore (Fig. 1*e, f*). The forespore, a germline cell, becomes the spore from which may arise subsequent progeny. The mother-cell is discarded when development of the spore is complete.

The transition from one stage to the next is governed by six regulatory proteins^{6,7} called sigma factors, which, by binding to RNA polymerase, determine which gene promoters are recognized⁸. These factors are the primary sigma factor of vegetative cells, σ^A , and five factors that become active during development called σ^H , σ^F , σ^E , σ^G and σ^K (in order of their appearance during sporulation). The σ^A and σ^H factors are active before the septum forms, and are not further considered. The other four, σ^F , σ^E , σ^G and σ^K , are specific to each cell type and direct gene transcription in one or the other compartment formed by septation.

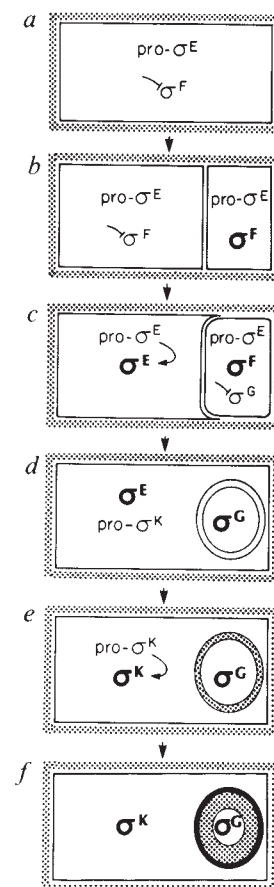
Establishment of cell type

Gene expression characteristic of the forespore is governed by the action of σ^F within that compartment shortly after the septum forms⁹. Although this factor is produced in the undivided sporangium¹⁰, and thus would be expected to be distributed to both compartments, the transcription of the genes under its control is delayed until after septation (P. Margolis and R.L., unpublished results) when the transcription it directs is confined to just one compartment⁹. This has been learned from experiments⁹ in which the *E. coli lacZ* gene is fused to a gene

under σ^F control; the location of β -galactosidase from the *lacZ* fusion is visualized by the use of antibodies against the enzyme and gold-conjugated secondary antibodies, followed by electron microscopy. Gold granules are almost exclusively located in the forespore (Fig. 2*a*).

Evidence that σ^F is produced before septation comes from the use of genetically mosaic sporangia in which the forespore chromosome is mutant for σ^F and the mother-cell chromosome is wild-type for σ^F (ref. 10). Such mosaic sporangia are obtained from cells mutant for σ^F by transforming them, at the start of sporulation, with wild-type DNA in conditions such that only one of the two chromosomes in the undivided sporangium is corrected¹¹. After septation, mutant and wild-type chromosomes are then randomly distributed between the forespore and the mother cell. Sporangia in which the forespore carries the mutant chromosome can sporulate, but the resulting spores are of genotype *spo*[−] (that is, the progeny of the spores are mutant for σ^F and cannot themselves sporulate)¹⁰. But σ^F is necessary for transcription in the forespore, meaning that forespores with mutant σ^F must have acquired some quantity of σ^F produced

FIG. 1 The stages of morphogenesis and a model for the compartmentalized action of sporulation sigma factors. (See text for an explanation of the morphological stages.) The stippled areas indicate the cell wall around the sporangia and the cortex between the forespore and mother-cell membranes (*e, f*). The heavy line around the forespore in *f* indicates the coat. Each sigma factor is in an inactive (light lettering) or active (bold lettering) state. The σ^F and σ^G factors are held inactive by an inhibitory protein (see text) as indicated by the symbol $\dashv$; the σ^E and σ^K factors are held inactive in the form of proproteins as indicated. The precise time at which σ^F and σ^E are activated is not known but is arbitrarily indicated as occurring just before, and at the start of, septum migration, respectively. For simplicity the inactive form of σ^F is omitted from the mother cell in *c* and pro- σ^E is omitted from the forespore in *d*. Also omitted are σ^A and σ^H , which are active before septation and whose activities are not compartmentalized.



from the wild-type chromosome before septation. Thus σ^F must be so controlled that its activity, but not its synthesis, is restricted to the forespore (Fig. 1a, b).

An important clue to this unknown mechanism comes from studies of the operon *spoIIA*, which consists of three cistrons *spoIIAA*, *spoIIAB* and *spoIIAC*, and in which σ^F is itself encoded^{12,13}. The product of the first cistron, SpoIIAA, is an inhibitor of that of the second¹⁴, SpoIIAB, which is in turn an inhibitor of σ^F (refs 14,15) (encoded by the third cistron¹⁶⁻¹⁸). Although the mode of action of SpoIIAB is not known, it probably inhibits σ^F directly rather than by interfering with (or 'repressing') access to target promoters^{9,14}.

How is the expression of the genes controlled by σ^F achieved selectively only in the forespore? One hypothesis⁹ is that SpoIIAA exists in inactive and active states, that it is inactive in the undivided cell and in the mother-cell after septation and that, in this state, it cannot prevent SpoIIAB from blocking the action of σ^F . Some feature of the post-septation sporangium must then drive SpoIIAA into the active state in the forespore only, inhibiting SpoIIAB and relieving σ^F from inhibition. Although the trigger for activation is not known, genetic experiments implicate the *spoIIIE* operon⁹, one of whose products is probably an integral membrane protein (P. Guzman, J. Westpheling and P. Youngman, personal communication) and which governs proper formation of the septum¹⁹ and may create the difference between the forespore and mother-cell compartments to which SpoIIAA responds.

As gene expression in the forespore is determined by σ^F , so gene expression in the mother cell is determined by the expression factor σ^E , which is similarly produced in the undivided sporangium¹⁰ and does not become active until after septation²⁰⁻²³. Thus immunoelectron microscopy²⁴ reveals that the transcription of a gene under the control of σ^E is chiefly confined to the mother-cell (Fig. 2b). Moreover, most if not all of the genes under the control of σ^E are required in, or have functions associated with, the mother-cell²⁵: σ^E is evidently a regulator or post-septation gene expression specific to the mother-cell.

For σ^E , the inactive differs from the active form by an extension of 27 amino acids at the N terminus²⁰ (C. P. Moran Jr, personal communication). In other words, σ^E is first produced as an inactive proprotein, pro- σ^E , the activation of which is catalysed by the gene product SpoIIGA of the first cistron of the operon *spoIIG* (refs 21, 26), whose second cistron (*spoIIGB*) is the structural gene for pro- σ^E (refs 27, 28). Interestingly, processing of pro- σ^E also requires the action of σ^F (and of all of the gene products required for the activation of that factor)^{21,22,29}. The inference is that an unknown gene under the control of σ^F is somehow required in the cleavage of pro- σ^E (see below).

The σ^E factor is not activated until after septation²⁰⁻²³, suggesting the attractive hypothesis that processing of pro- σ^E is

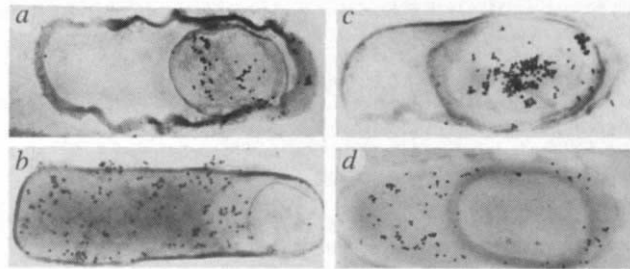


FIG. 2 Use of immunodecorated thin sections and electron microscopy to demonstrate compartmentalized gene expression. Thin sections of sporangia at intermediate (a, b) or late (c, d) stages of sporulation were stained with antibodies to β -galactosidase and with gold-conjugated secondary antibodies. Each sporangium contained *lacZ* fused to a gene under the control of σ^F (a), σ^E (b), σ^G (c) or σ^K (d). The experiment was done as described^{9,24}, except that the size of the gold granules was increased by silver enhancement using the IntenSE M kit (Janssen Biotech).

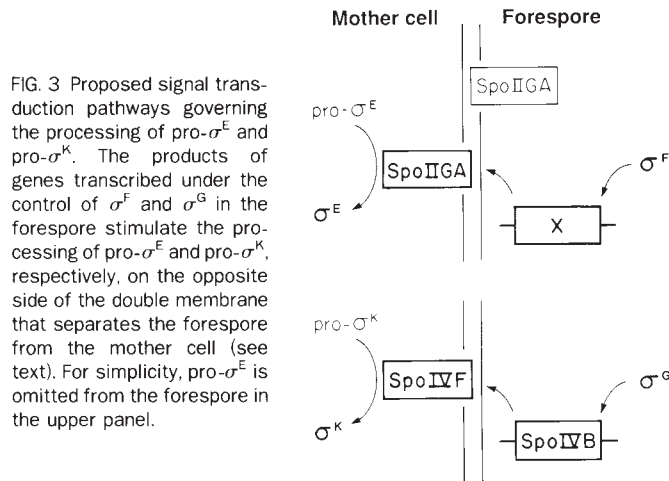


FIG. 3 Proposed signal transduction pathways governing the processing of pro- σ^E and pro- σ^K . The products of genes transcribed under the control of σ^F and σ^G in the forespore stimulate the processing of pro- σ^E and pro- σ^K , respectively, on the opposite side of the double membrane that separates the forespore from the mother cell (see text). For simplicity, pro- σ^E is omitted from the forespore in the upper panel.

confined to the mother-cell²⁵ (Fig. 1c). It is also possible that pro- σ^F might be processed in both compartments, but that some other mechanism might limit the action of σ^E to the mother-cell. Reports of experiments involving the fractionation of the contents of the sporangium have indeed suggested that mature σ^E is equally present in the two compartments³⁰, but the experiments are technically difficult and the results may not be conclusive.

If it is true that mature σ^E is generated only in the mother-cell, what might be the mechanism? It is unlikely that the putative processing enzyme SpoIIGA or its substrate pro- σ^E can be compartmentalized: the operon *spoIIG* is switched on before septation¹⁰, so that both its products are likely to be present in both compartments afterwards. But the observation^{21,22,29} that processing of pro- σ^E requires the action of the forespore transcription factor σ^F suggests this model of selective processing in the mother-cell: the product of an unidentified gene under the control of σ^F sends a signal across the membrane to the mother-cell, where it stimulates the processing of pro- σ^E (refs 9, 24; Fig. 3) and, because the stimulus is directional, processing takes place only in the compartment opposite that in which σ^F is active. This hypothesis is attractive because a similar directional signal controls the activity of a second transcription factor later in development.

Switch to late-acting sigmas

There are two phases in the compartmentalized expression of the sporulation genes, of which the early phase is governed by σ^F and σ^E . While the set of genes whose transcription is controlled by σ^F is still poorly characterized^{14,15,31,32}, that controlled by σ^E includes genes involved in the engulfment of the forespore and in the early steps of cortex and coat formation³³⁻³⁷. Gene expression in the late phase, on the other hand, which begins with engulfment (Fig. 1d) is controlled by the transcription factors σ^G and σ^K (refs 18, 38, 39), which are true compartment-specific regulatory proteins in that their synthesis and site of action is confined to one or the other cell type^{24,38,40,41}.

Specifically, σ^G governs the transcription of genes expressed in the forespore, which include genes encoding a family of small acid-soluble proteins which are abundant in the forespore⁴². Similarly, σ^K is the chief determinant of gene expression in the late-phase mother-cell, directing the transcription of genes encoding the most abundant proteins of the spore coat^{37,43,44} and one or more genes involved in the formation of the cortex⁴⁵. Immunodecoration demonstrates that gene expression directed by σ^G and σ^K is restricted to the forespore and the mother-cell respectively^{24,41} (Fig. 2c, d).

The switch from early to late-phase expression requires not only mechanisms for turning on the synthesis of the late-phase σ -factors but also to remove the early-phase σ -factors from the RNA polymerase. The removal of the early-phase mother-cell factor σ^E may be explained by the observation that σ^E (but not pro- σ^E) is rapidly proteolysed when not bound to core RNA

polymerase (as, for example, after the initiation of a cycle of transcription)⁴⁶; mother-cell depletion of σ^E could thus result from the inherent instability of the factor coupled with cessation of the synthesis of σ^E . As yet, it is not known whether the forespore factor σ^F is physically depleted or, alternatively, simply displaced from RNA polymerase by newly synthesized σ^G .

How is compartment-specific synthesis of the late-phase sigma factors achieved? In the forespore, σ^G is selectively synthesized because^{14,15,32} its structural gene (*spoIIIG*) is controlled by σ^F (Fig. 1c). But, remarkably, σ^G is subject to a second kind of control acting at the level of its activity^{38,47}: although the structural gene for σ^G is induced shortly after septation, transcription of the genes it controls is delayed until the forespore is fully engulfed (Fig. 1d). (The mechanism of this inhibition is not known, but genetic experiments⁴⁸ implicate SpoIIAB, which may therefore govern the activity of both forespore transcription factors.) But once the activity of σ^G has been unleashed, it maintains and amplifies its own synthesis by recognizing the promoter for its own structural gene.

By contrast, the mother-cell late-phase factor σ^K is controlled by three distinct regulatory mechanisms (Fig. 4), of which the first and most extravagant (but least important) involves a DNA rearrangement in the mother-cell chromosome to form its structural gene, called *sigK*^{49,50}. What happens is that site-specific reciprocal recombination effects in-frame joining of partial genes encoding the amino- and carboxy-terminal portions of σ^K . The rearrangement entails the excision as a circle of an intervening DNA element (42 kilobases long) called *skin* (ref. 50) and is catalysed by the product of a recombinase gene (*spoIVCA*) which is itself located on the same intervening element^{50,51} (Fig. 4). (The gene product SpoIVCA belongs to the Hin, Pin, Gin, TnpR family of site-specific recombinases⁵¹.) The rearrangement also requires a small DNA-binding protein, SpoIID, which may be part of the synaptic complex⁵⁰. The σ^K gene rearrangement is confined to the mother-cell compartment because the transcription of both the *spoIVCA* recombinase gene (Y. Kobayashi, personal communication) and that (*spoIID*) for the DNA-binding protein^{52,53} (C.P. Moran, personal communication) is controlled by the early mother-cell factor σ^E .

Evidently the rearrangement need not be, and is not, reversible; both the mother-cell and its chromosome are discarded when the spore has matured. But the *skin* element is not normally excised either in the vegetative cell or in the forespore. It is interesting that the rearrangement as such is not essential for the proper compartmentalization of gene expression; the growth and development of cells in which the whole *skin* element has been deleted are not impaired although both mother-cell and forespore carry an intact copy of *sigK* from the onset of sporulation⁵⁰. It is relevant that in *B. thuringiensis*, the gene corresponding to *sigK* is not interrupted by an intervening element⁵⁴.

In the second level of control, transcription of the intact *sigK* gene is directed by σ^E in conjunction with SpoIID, which in

this context is a positive regulatory protein^{39,40} (Fig. 4). Once again, because σ^E and SpoIID are specific to the mother-cell, this arrangement ensures that the transcription of *sigK* is also specific to the cell type carrying the reconstituted gene.

The third level of control is the conversion of σ^K from an inactive precursor^{39,55,56} (Fig. 1e and 4). As with σ^E , the primary product of *sigK* is a proprotein, in this case with an amino-terminal extension of 20 amino-acids³⁹. The conversion of pro- σ^K to mature protein is governed by the *spoIVF* operon, which encodes either the processing enzyme or its regulator^{56,57}, and which is transcribed at an early stage in the mother-cell under control of σ^E (ref. 57).

Just as the transcription of the gene for σ^G is auto-catalytic, so is that of *sigK*, which is regulated positively by its product σ^K , again in conjunction with SpoIID (ref. 39). Thus the compartment-specific synthesis of a transcription factor is again maintained and amplified by itself. But mature σ^K also directs the transcription of the regulatory gene *gerE*, encoding a small DNA-binding protein which acts in conjunction with σ^K to direct the transcription of a class of mother-cell genes expressed late in the existence of the mother-cell^{37,43}. Thus the mother-cell hierarchy of gene regulation is more complex than that of the forespore, which involves just two known regulatory proteins, entailing the successive appearance of two sigma factors and two DNA binding proteins in the order σ^E , SpoIID, σ^K and GerE (ref. 37).

Intercompartmental communication

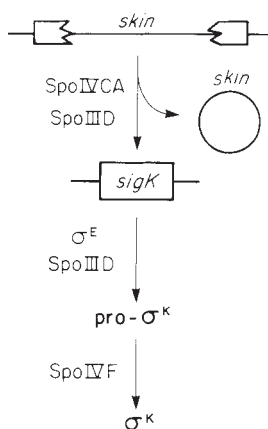
The expression of genes in the forespore and mother-cell is not independent, but is coordinated by intercompartmental communication coupling the appearance of a sigma factor in one compartment to the activity of an earlier sigma factor in the other. Thus the appearance in the mother-cell of σ^E by the activation of the pro-protein depends on the activity of σ^F in the forespore. Likewise, the activity of σ^G in the forespore waits and evidently depends upon the engulfment of the forespore by the mother-cell, which is brought about by the action of genes under the control of σ^E , notably *spoIID* (whose product is required for pinching off the forespore as a free protoplast^{51,19}) and the products of the *spoIIIA* operon (some of which are membrane-bound proteins: A.M. Guérout-Fleury and P.S., unpublished results) presumed to act from outside the forespore to relieve the inhibition of σ^G activity therein⁴⁷.

The coordination of gene expression in the two compartments is further illustrated by the coupling of the appearance of σ^K in the mother-cell to the action of σ^G in the forespore^{45,55,56}, which involves the following signal-transduction pathway: σ^G turns on the transcription of the forespore gene *spoIVB* whose product stimulates the activity in the mother-cell of the SpoIVF proteins, which in turn govern the processing of pro- σ^K (ref. 45; Fig. 3). The SpoIVF proteins are believed to be embedded in the outer membrane surrounding the forespore, and to be activated by the appearance in the forespore of SpoIVB (ref. 57).

Support for this view has been provided by the isolation of mutations in *B. subtilis* in which the dependence of active σ^K on the activity of σ^G has been removed; both gain-of-function mutations of *spoIVF* and a deletion of the amino acid sequence distinguishing pro- σ^K from the active protein have been obtained⁵⁶. In both cases, transcription of genes under the control of σ^K begins an hour earlier than normally, and, as an apparent consequence, spore formation is impaired⁵⁶. This demonstrates that the timing of late gene expression in the mother-cell hangs on the link between the activation of pro- σ^K and the activity of σ^G in the forespore.

An interesting evolutionary speculation now arises. The activation of σ^K by inter-compartmental signalling is reminiscent of the proposed coupling of σ^E activation in the mother-cell to forespore gene expression directed by σ^F (Fig. 3). Could it be that the mechanism linking pro-sigma processing in one cell to gene expression in the other directed by a different transcription

FIG. 4 Three levels of control of σ^K synthesis. The figure depicts the control of σ^K synthesis at the levels of DNA rearrangement, transcription, and proprotein processing (see text). The boxes with serrated edges at the top of the figure represent the two half-coding elements for σ^K in the chromosome before the DNA rearrangement.



factor has been duplicated in the course of evolution? It is interesting that σ^G strongly resembles σ^F and σ^K , σ^E . Perhaps an ancestor of *B. subtilis* relied on σ^F and σ^E alone to govern the differentiation of mother-cell and forespore⁵⁸.

Crisscross regulation

The differential expression of genes in the forespore and mother-cell thus involves four σ -factors, each specific to a cell type. The first to act is σ^F , synthesized in the undivided sporangium but activated only after septation. That switches on transcription of a gene whose product may lead to the appearance of active σ^E in the mother-cell. Although σ^F also switches on transcription in the forespore of the structural gene for σ^G , gene expression directed by that factor does not begin until engulfment, which is driven by genes under control of σ^E in the mother-cell. The factor σ^E also brings about the transcription of the rearranged gene for σ^K , the last in the sequence, but the appearance of mature σ^K depends on the activation of pro- σ^K which is controlled by σ^G in the forespore. The compartmentalized action of all these factors ultimately derive from whatever restricts the activity of the first factor in the network, σ^F , to the forespore.

Thus the actions of four transcription factors are linked together in two different ways (Fig. 5). First, at the level of transcription, there are two parallel pathways in which σ^F directs the transcription in the forespore of the structural gene for σ^G and in which σ^E directs the transcription in the mother-cell of the gene for σ^K (together with the others involved in its production). Second, gene expression in the two cell types is linked in a crisscross fashion by mechanisms operating at the level of, and coordinating, the activities of the four factors: the selective activity of σ^F in the forespore signals the appearance of σ^E in the mother-cell, the activity of which regulates that of σ^G in the forespore, which in turn determines the appearance of σ^K in the mother-cell.

The parallel pathways ensure that the early factors σ^F and σ^E are replaced by the late-phase factors σ^G and σ^K respectively, while the crisscross pathway links all four factors in such a way that their sequential activity in the two cell-types is tightly coupled.

In the crisscross pathways, signals go from one cell to the other, transverse the double membrane separating the two cell types. Thus, the cells communicate by telling each other when to proceed to the next development stage. In each case signalling seems to be determined by specific morphological cues: asym-

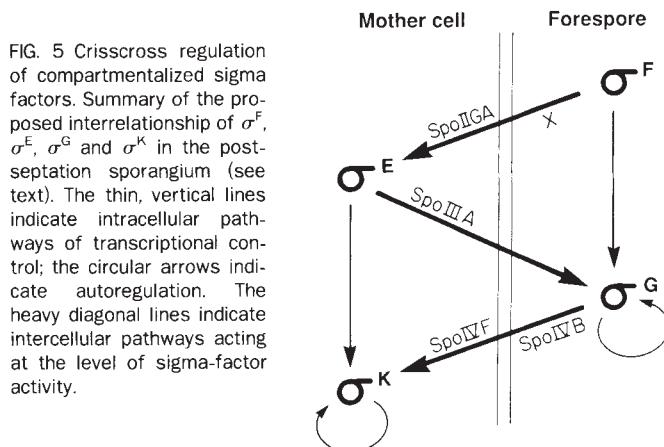


FIG. 5 Crisscross regulation of compartmentalized sigma factors. Summary of the proposed interrelationship of σ^F , σ^E , σ^G and σ^K in the post-septation sporangium (see text). The thin, vertical lines indicate intracellular pathways of transcriptional control; the circular arrows indicate autoregulation. The heavy diagonal lines indicate intercellular pathways acting at the level of sigma-factor activity.

metric septation leads to activation of σ^F in the smaller compartment; some subtle feature of the sporulation septum created by the action of σ^F stimulates the processing of pro- σ^E ; the engulfment of the forespore driven by the products of genes controlled by σ^E appears to activate σ^G ; the free forespore protoplast and a signal transmitted across it by the action of σ^G stimulates the processing of pro- σ^K . None of the signalling processes is fully understood, but according to this point of view each sigma factor is tightly controlled by a series of checkpoints that tie its activation to the completion of a landmark event in the course of morphogenesis.

The generation of differentiated cell types is a general feature of development, but the molecular mechanisms by which cell specialization is achieved differ widely among different organisms and among different cell types in the same organism. Nevertheless, the principles that have emerged from the study of differentiation in *B. subtilis*, namely the compartmentalization of transcription factors, the regulation of these factors by intercellular communication, and the linkage of the factors to morphological checkpoints, are likely to be common themes in developing systems of many kinds. □

Richard Losick is in the Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138, USA; Patrick Stragier is in the Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France.

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Electron microscopy at 1-Å resolution by entropy maximization and likelihood ranking

W. Dong*, T. Baird*, J. R. Fryer*[†], C. J. Gilmore^{†‡}, D. D. MacNicol[‡], G. Bricogne^{§†}, David J. Smith[¶], M. A. O'Keefe^{||} & S. Hövmöller[#]

* Electron Microscope Centre, Chemistry Building, University of Glasgow, Glasgow G12 8QQ, UK

[†] Chemistry Department, University of Glasgow, Glasgow G12 8QQ, UK

[§] MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

[¶] Center for Solid State Science, Arizona State University, Tempe, Arizona 85287, USA

^{||} National Center for Electron Microscopy, University of California, LBL, Berkeley California 94720, USA

[#] Structural Chemistry, University of Stockholm, S-10691, Stockholm, Sweden

The resolution of electron microscopy may be extended by combining the phase information in microscope images with electron diffraction intensities. A general method for obtaining structural reconstructions at a resolution greater than that of the phase information is demonstrated for crystals of perchlorocoronene. By using entropy maximization methods combined with likelihood ranking, the resolution is extended from 0.32 nm in the microscope images to 0.1 nm in the reconstruction from phase and intensity data.

orthorhombic, space group *Cmca* with $a = 2.22$, $b = 0.82$, $c = 1.29$ nm. The calculated density is 2.01 g cm^{-3} , and there are 4 molecules per unit cell. The molecule is not flat, although the 12 central carbon atoms form a planar moiety. In the *a*-projection the perchlorocoronene molecule presents two concave faces with pairs of chlorine atoms (and directly attached carbon atoms) alternately disposed above and below the mean molecular plane. The epitaxial film had the same crystal parameters as the bulk single crystal and was assumed to be the same polymorph. It was oriented to show the *b*-*c* projection, and Fig. 1*b* shows a molecule in this orientation, as determined from the X-ray structure. The stacking sequence of the molecules can be seen in the inset of Fig. 5.

Experimental

Perchlorocoronene was sublimed under vacuum onto a KCl(100) substrate, maintained at 200°C , in amounts that ensured that the film thickness did not exceed 10 nm. The film was reinforced with carbon and then mounted on an electron microscope grid. The high-resolution electron microscope at Cambridge was used at an operating voltage of 500 kV. Minimal exposure, and the use of an image intensifier, enabled images to be obtained with a minimum of radiation damage¹⁰. Many images of different areas of the specimen were scanned to increase the opportunity of obtaining one at optimum defocus, as it is not possible to record a focal series with radiation-sensitive specimens. Images were recorded on standard electron microscope film, and developed under normal conditions. Figure 2*a* shows an image in which the lattice fringes are resolved. The molecular projection is manifest in the herring-bone structure intersecting the main lattice periodicities. This image was typical in that there was buckling and bending in the crystal caused by radiation damage, shown by the changes in contrast. The electron diffraction pattern shown in Fig. 2*b* had the expected 2-fold symmetry, and patterns were measured at different exposures so that the intensities of low- and high-order reflections could be measured within the dynamic range of the film.

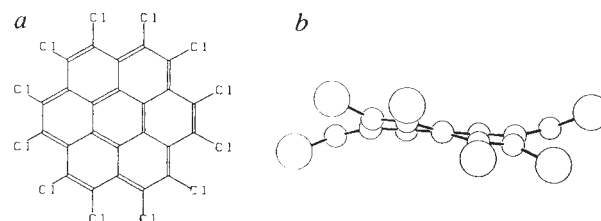


FIG. 1 *a*, Structure of perchlorocoronene, $\text{C}_{24}\text{Cl}_{12}$. *b*, The molecule in the *b*-*c* projection as imaged in the microscope. In this projection only the out-of-plane chlorine and peripheral carbon atoms are distinguished. The large circles represent two superimposed chlorine atoms, and the small circles superimposed carbon atoms. The lines are the superimposition plane across the molecule.

AN electron diffraction pattern can be obtained from a crystal with a maximum dimension of only 50 nm. The same crystal can also yield direct images whose Fourier analysis provides phase information for some of the diffraction intensities. Combination of these intensities and phase allows us to construct the object's molecular and crystal structure, at a resolution limited to that with which phases can be extracted from the microscopic images. In contrast, X-ray diffraction is limited to much larger crystals and loses all phase information. In practice, however, electron microscopy has an image resolution limited to 0.16 nm, even for a thin crystal under optimum conditions, and the accuracy of the phases and intensities at this resolution is low. Organic specimens degrade rapidly in the electron beam; even for those that are most resistant to radiation, the halogenated aromatics, a direct image resolution of only 0.2–0.3 nm is possible^{1,2}. Unfortunately, at this resolution, neither the image nor a reconstruction using experimental phases and intensities is sufficient to determine the conformation of a small molecule, and larger molecules such as macromolecules and polymers are much more sensitive to the electron beam. We have synthesized a very radiation-resistant polycyclic molecule, perchlorocoronene³, $\text{C}_{24}\text{Cl}_{12}$, prepared it as an epitaxial film⁴, and obtained images and diffraction patterns at room temperature. Many images were recorded under minimum exposure conditions, and those images close to Scherzer defocus at 500 keV used for processing. The phased intensities have a resolution of 0.3 nm, and we extended the resolution to 0.1 nm by applying a technique combining entropy maximization, a tree-directed phase search, and likelihood estimation⁵ previously used for X-ray single-crystal and powder diffraction data^{6–9}.

Structure and orientation of specimen

Figure 1*a* and *b* shows the structure of perchlorocoronene as determined by single-crystal X-ray diffraction³; these results were used to check the structure derived here. The crystals are

[†] Correspondence should be addressed to J.R.F. or C.J.G. G.B. is also at LURE, Bâtiment 209D, 91405 Orsay, France.

Image processing and simulation

The experimental images and diffraction patterns were digitized with a Joyce Loebble microdensitometer. We also recorded some diffraction patterns by scanning the film with a TV camera and measuring intensities using the SEMPER image-processing system (Synoptics Ltd). The experimental intensities showed only small deviations, which may have been caused by dynamical scattering, and the dimensional accuracy was rarely better than 1%. The phases were calculated from the transform of the digitized image and the phase origin fitted to a unit cell vertex (R. Henderson, personal communication). The phases down to the resolution limit of the image were adjusted to 0 or π to fit the centrosymmetric projection of the crystalline film in accordance with the space group¹¹. Diffraction intensities to the same resolution as the phase information (about 0.3 nm; see below) were combined with the phase information to give a structural solution shown in Fig. 3. This represents the limit that could be obtained by conventional microscopy, and uses the plane symmetry obtained from the electron diffraction pattern. The result is not very reliable, because of the inaccuracies in the intensities.

The X-ray data provided the necessary information for multi-slice dynamical calculations using the SHRL1 computer program¹², and parameters such as crystal thickness, microscope resolution and objective-lens defocus could be explored. The projected potential of the specimen was calculated using 2,075 structure factors extending to 0.025-nm resolution. Simulated images were calculated from this potential for a range of

resolutions assuming perfect lens characteristics, and were used for comparison with the structure derived from the phases determined from maximum entropy. Simulated images were also calculated for the experimental microscope parameters, and these were used to assess the quality and resolution of the experimental image. Resolution was defined by the spatial frequency corresponding to the highest-order reflection included in the computation. The experimental resolution (Scherzer) of which the microscope was capable was 0.18 nm, but because of radiation damage, the best image which could be obtained had 0.32-nm resolution (Fig. 4; simulated image inset).

Theoretical basis of phase extension

The 0.3-nm-resolution phase information obtained by image analysis would usually be extended to the 0.1-nm-resolution set of diffraction intensity measurements by building a molecular model from the electron density map obtained from the optical data (of which Fig. 3 is an example), and refining it against the unphased intensity observations between 0.3 and 0.1 nm. This procedure has two drawbacks: many initial models could account for the limited number of features in Fig. 3, and the functional relation between the model coordinates and the extra intensity observations is nonlinear. Such an approach would therefore involve an extensive global search through a space of hypothetical structures before it could use a conventional (local) refinement of a trial structure. Alternatively one can attempt a phase extension in reciprocal space by direct methods¹³, but stability can be a problem when the diffraction data are incomplete.

Instead we use a search procedure first proposed by Bricogne⁵ and established⁶ through successful applications to crystals of small molecules⁷, powders⁸ and a protein⁹. A space of hypothetical phase sets is explored in a hierarchical fashion by building a search tree^{5,6}, similar to those used in game-playing computer programs, and each set ranked according to a statistical criterion (in this case the log-likelihood gain).

The quantitative theory, described elsewhere^{5,14-16}, is based on the same assumptions as traditional direct methods of phase determination¹⁷⁻¹⁹, namely that the ensemble of crystal structures within which the solution is sought may be generated by placing atoms randomly and independently of each other in the asymmetric unit of the crystal. From limit theorems of probability theory, we can then estimate the joint probability distribution of the corresponding structure factors, and substitute observed amplitudes into them to obtain conditional joint distributions

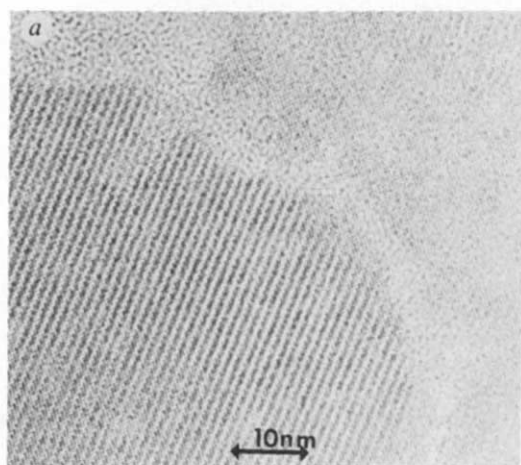


FIG. 2 *a*, Electron micrograph of perchlorocoronene supported on a carbon film. Lattice fringes are visible. *b*, Electron diffraction pattern (500 keV) from an epitaxial film, showing the expected 2-fold symmetry.

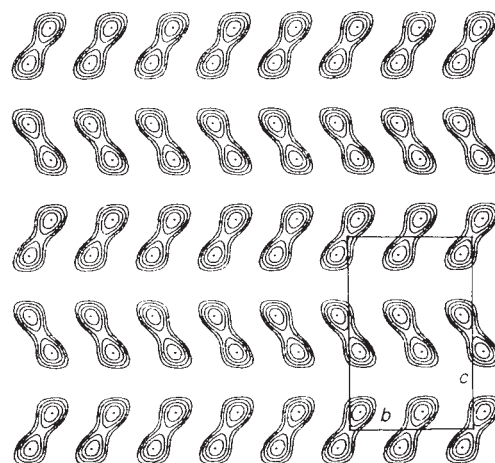


FIG. 3 Structure of the projection down *a* of perchlorocoronene calculated from experimental phases and intensities at 0.32-nm resolution. The unit cell outline is shown.

TABLE 1 Normalized diffraction data from perchlorocoronene

Serial number	<i>k</i>	<i>l</i>	$ U_{\mathbf{h}}^{\text{obs}} $	Resolution (nm)
1	2	1	0.25	0.20
2	0	4	0.25	0.32
3	1	3	0.25	0.30
4	1	2	0.20	0.35
5	3	4	0.19	0.13
6	1	0	0.14	0.41
7	2	0	0.13	0.21
8	0	2	0.13	0.65
9	1	6	0.12	0.19
10	1	1	0.11	0.39
11	3	0	0.08	0.14
12	0	8	0.08	0.16
13	3	5	0.07	0.12
14	0	6	0.06	0.22
15	2	10	0.06	0.11
16	1	11	0.06	0.11
17	2	8	0.05	0.13
18	1	7	0.05	0.17
19	2	2	0.04	0.20
20	2	3	0.04	0.19
21	2	6	0.03	0.15
22	1	5	0.03	0.22
23	1	4	0.03	0.25
24	4	0	0.03	0.10
25	1	12	0.03	0.10
26	3	7	0.02	0.11
27	2	5	0.02	0.16
28	4	1	0.02	0.10
29	3	1	0.02	0.14
30	2	4	0.02	0.17
31	4	2	0.02	0.10
32	2	7	0.02	0.14
33	2	9	0.02	0.12
34	3	2	0.01	0.13
35	1	8	0.00	0.15
36	3	3	0.00	0.13
37	1	9	0.00	0.14
38	1	10	0.00	0.12
39	3	6	0.00	0.12

Serial number, *k* and *l* indices (*h*=0), unitary structure factor magnitude ($|U_{\mathbf{h}}^{\text{obs}}|$) and resolution (nm) for perchlorocoronene diffraction data.

for the phases. The latter indicate that certain combinations of phases are more probable than others, and hence contain phase information.

Traditionally the Edgeworth series^{18,19} has been used to approximate joint distributions, but this is unsuitable for large structure-factor amplitudes^{5,16}. In addition, the method assumes the distribution of the random atomic positions to be uniform. These limitations can be overcome simultaneously^{5,16} by using the saddlepoint method^{20,21} instead of the Edgeworth series; this always yields optimal estimates of joint probabilities involving large amplitudes, and is equivalent to requiring that the *a priori* distribution of random atomic positions should be updated whenever phase assumptions are made so as to have maximum entropy under the constraints embodied in these assumptions. This does not amount to a wholesale adoption of the maximum entropy principle (see ref. 16 for a critical discussion of this point).

The diffraction intensities are normalized using conventional methods¹⁹ to give unitary structure-factor amplitudes $|U_{\mathbf{h}}^{\text{obs}}|$. The *U*s are split into two sets: the basis set $\mathbf{H} = \{\mathbf{h}_1, \mathbf{h}_2, \dots, \mathbf{h}_n\}$ comprising the low-resolution phased diffraction intensities with associated phase angles $\Phi = \{\varphi_1, \varphi_2, \dots, \varphi_n\}$ obtained from the Fourier transform of the optical image(s) of the crystal, and the disjoint set $\mathbf{K}$ comprising the unphased reflections of higher

resolution. Only the symmetry-unique reflections are used. The distribution $q_{\mathbf{H},\Phi}^{\text{ME}}$ having maximum entropy (ME) compatible with these intensities and phases is constructed by maximizing the relative entropy

$$S_m(q) = - \int_V q(\mathbf{x}) \log [q(\mathbf{x})/m(\mathbf{x})] d^3\mathbf{x} \quad (1)$$

(where *V* is the unit cell of volume *vol* (*V*), and *m*(**x**) = 1/*vol* (*V*)) under the constraints:

$$\int_V q(\mathbf{x}) \exp(2\pi i \mathbf{h} \cdot \mathbf{x}) d^3\mathbf{x} = |U_{\mathbf{h}}^{\text{obs}}| \exp(i\phi_{\mathbf{h}}) \quad \text{for all } \mathbf{h} \in \mathbf{H} \quad (2)$$

That is, $q_{\mathbf{H},\Phi}^{\text{ME}}(\mathbf{x})$ must reproduce the intensities and phases of the reflections used in its generation. It has Fourier coefficients with non-negligible amplitude $|U_{\mathbf{k}}^{\text{ME}}|$ for many non-basis-set reflections $\mathbf{k} \notin \mathbf{H}$, especially when **k** is in the second neighbourhood of **H**, consisting of the reflections $\mathbf{h}_1 \pm {}^t\mathbf{R}_{\mathbf{g}} \cdot \mathbf{h}_2$ for $\mathbf{h}_1, \mathbf{h}_2 \in \mathbf{H}$, where ${}^t\mathbf{R}_{\mathbf{g}}$ is the transpose of a rotation matrix obtained of the crystal space group. This is the phenomenon of maximum entropy extrapolation, and it causes the conditional distribution of $|U_{\mathbf{k}}|$ to become a Rice distribution^{5,6,14} rather than the gaussian or Rayleigh distributions that occur in the Wilson statistics of traditional direct methods (and correspond to $|U_{\mathbf{k}}^{\text{ME}}| = 0$). This distortion depends on assumptions as to the phases of the basis set, and can be detected^{5,6,8,14,15} by calculating the log-likelihood gain:

$$L = \sum_{\mathbf{h}} \log \frac{P(|U_{\mathbf{h}}^{\text{obs}}| | U_{\mathbf{h}_j} = |U_{\mathbf{h}}^{\text{obs}}| \exp(i\phi_{\mathbf{h}_j}), j = 1, \dots, \nu)}{P(|U_{\mathbf{h}}^{\text{obs}}| | U_{\mathbf{h}_j} = 0, j = 1, \dots, \nu)} \quad (3)$$

L will be largest when the phase assumptions for the basis set lead one to predict deviations from the Wilson distribution in the unphased amplitudes $|U_{\mathbf{k}}|$, $\mathbf{k} \in \mathbf{H}$, that most closely match the distribution of the measurements $|U_{\mathbf{k}}^{\text{obs}}|$.

A tree-directed search through all possible combinations of phases is now initiated by successively enlarging the basis set of reflections by adding data from set **K**. Because the phases of these reflections are unknown, they are given permuted values. For centric reflections (always the case here) the permutations are 0 and π ; for acentrics we use $\pm\pi/4, \pm3\pi/4$. At each stage the various phase assumptions Φ for these reflections are tested by numerically constructing $q_{\mathbf{H},\Phi}^{\text{ME}}$, and calculating the log-likelihood gain *L* resulting from the maximum entropy extrapolation outside **H**. *L* is then used as a heuristic function to prune the search tree, retaining only those nodes with the highest *L* values. This essentially a Bayesian method^{5,6,14-16} in which *L* alone is considered because it is more sensitive than $S_m(q_{\mathbf{H},\Phi}^{\text{ME}})$ to the phases in Φ , although the entropy can be useful as a secondary indicator.

At any stage of the search, a centroid map can be calculated⁶ for a given node of the tree, using as Fourier coefficients

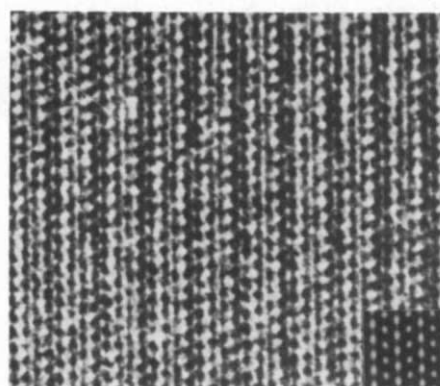


FIG. 4 Experimental image (500 keV) of perchlorocoronene with an inset of the computed image at 0.32-nm resolution.

$U_{\mathbf{h}} = |U_{\mathbf{h}}^{\text{obs}}| \exp(i\phi_{\mathbf{h}})$ for $\mathbf{h} \in \mathbf{H}$, and $U_{\mathbf{k}} = \langle |U_{\mathbf{k}}| \exp(i\phi_{\mathbf{k}}) \rangle$ for $\mathbf{k} \notin \mathbf{H}$, where the mean value is calculated with respect to the conditional distribution

$$P(|U_{\mathbf{h}}^{\text{obs}}| \exp(i\phi_{\mathbf{h}}) | U_{\mathbf{h}_j} = |U_{\mathbf{h}_j}^{\text{obs}}| \exp(i\phi_{\mathbf{h}_j}), j = 1, \dots, \nu)$$

Practical aspects of phase extension

The diffraction data were normalized using the MITHRIL²² computer program to give unitary structure-factor magnitudes ($|U_{\mathbf{h}}^{\text{obs}}|$) and their standard deviations, $\sigma_{\mathbf{h}}$, using electron scattering factors and imposing an overall isotropic temperature factor of 0.01 nm². Table 1 lists the normalized data. There were six phases available at a resolution of 0.3 nm from the Fourier transform of the optical image: these were reflections 2, 3, 4, 6, 8 and 10 in Table 1. These phases and their associated U -magnitudes were used to form the initial basis set $\mathbf{H}$, and

constrained entropy maximization produced a ME distribution $q^{\text{ME}}(\mathbf{x})$. To allow for measurement errors, we do not aim for a perfect fit between $|U_{\mathbf{h}}^{\text{obs}}|$ and $|U_{\mathbf{h}}^{\text{ME}}|$; rather, we use a reduced χ^2 statistic:

$$\chi^2 = 1/n \sum_{\mathbf{h} \in \mathbf{H}} 1/\sigma_{\mathbf{h}}^2 (|U_{\mathbf{h}}^{\text{obs}}| - |U_{\mathbf{h}}^{\text{ME}}|)^2$$

where n is the total number of degrees of freedom (for a centrosymmetric space group, this is the number of reflections in $\mathbf{H}$), and we aim at a target value of $\chi^2 = 1.0$. In this way node 1 of a phasing tree was generated; the corresponding centroid map was similar to Fig. 3 with only the general outline features of the molecule visible. The complete phasing tree is summarized in Table 2.

Using a selection algorithm based on optimal second-neighbourhood enlargement of the basis set, we added two new reflections ($\mathbf{h} \in \mathbf{K}$, $|U_{\mathbf{h}}^{\text{ME}}| \approx 0$) with serial numbers 7 and 9 to the basis set. As their phases were unknown, all possible combinations of 0° and 180° were used, thus generating four new nodes (numbered 2–5) on the phasing tree. Each node was subjected to constrained entropy maximization as for node 1.

The four nodes generated from node 1 had log-likelihood gains in the range –0.1 to 0.0. This variation is small, and accordingly all four were retained and two further reflections, 5 and 18, were given permuted phases, thus generating nodes 6–21. The L -values ranged from –0.01 to 2.21. The node with the highest likelihood, number 8, produced a centroid map in which the molecular boundary was clear, and with a resolution showing atomic detail. The best two nodes were retained, and reflections 13 and 29 were permuted to generate nodes 22–29. Reflection 29 has $|U_{\mathbf{h}}^{\text{obs}}| = 0.02$; conventional crystallographic direct methods would be unable to determine the phase of an intensity as weak as this, but the log-likelihood gains for nodes 22–29 show that the maximum entropy method can indeed determine the phase of such reflections. Only two nodes, 24 and 28, have $L > 1.0$, and these were kept. Reflections 15 and 16 were then permuted to generate nodes 30–37; two nodes were retained and two weak reflections 28 and 36 given permuted phases, thus generating the final nodes 46–53 of which number 53 had the highest log-likelihood gain.

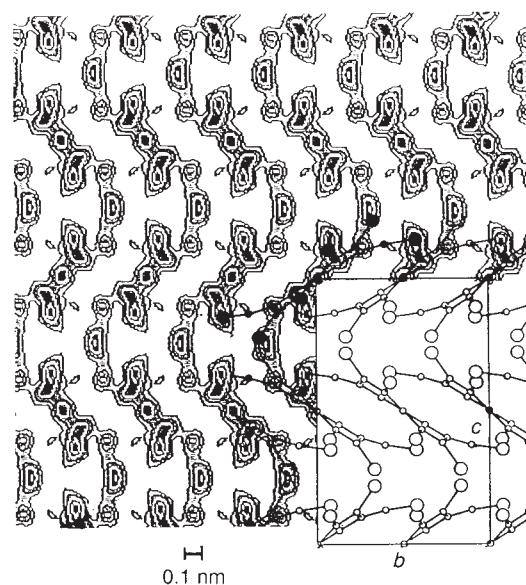


FIG. 5 Centroid map from node 53 of the phasing tree. Inset: molecular array on the b - c face of the unit cell. To assist in recognition, the molecules are extended beyond the unit cell, and the molecule whose centre is at the top left-hand corner of the unit cell has atoms shaded where they overlap the centroid map. The coordinates are derived from the X-ray structure.

TABLE 2 Phasing tree for perchlorocoronene

Node	To node	Entropy	Log likelihood gain
1		–0.21	0.00
2	1	–0.16	–0.10
3	1	–0.19	–0.09
4	1	–0.24	0.00
5	1	–0.24	0.00
6	2	–0.31	1.32
7	2	–0.22	0.56
8	2	–0.32	2.21
9	2	–0.23	0.50
10	3	–0.38	1.12
11	3	–0.17	0.03
12	3	–0.39	1.42
13	3	–0.23	0.06
14	4	–0.26	0.66
15	4	–0.21	0.53
16	4	–0.29	1.61
17	4	–0.21	0.54
18	5	–0.32	0.30
19	5	–0.18	–0.01
20	5	–0.33	0.07
21	5	–0.30	0.20
22	8	–0.25	0.16
23	8	–0.27	0.17
24	8	–0.30	1.23
25	8	–0.24	–0.60
26	16	–0.24	0.26
27	16	–0.28	0.36
28	16	–0.28	1.21
29	16	–0.32	0.16
30	24	–0.28	1.58
31	24	–0.25	0.67
32	24	–0.26	1.07
33	24	–0.23	0.25
34	28	–0.28	2.00
35	28	–0.23	0.61
36	28	–0.25	1.04
37	28	–0.21	0.16
38	30	–0.27	2.76
39	30	–0.30	3.85
40	30	–0.27	2.75
42	30	–0.28	3.18
42	34	–0.27	2.97
43	34	–0.27	3.59
44	34	–0.26	2.53
45	34	–0.26	2.96
46	39	–0.27	4.10
47	39	–0.28	4.17
48	39	–0.29	4.47
49	39	–0.29	4.28
50	43	–0.24	3.13
51	43	–0.25	3.81
52	43	–0.26	3.98
53	43	–0.27	4.59

The centroid map for node 53 is shown in Fig. 5. The resolution exceeds 0.1 nm and is devoid of spurious peaks. Inset into Fig. 5 is a *b*-*c* projection of the molecules in the unit cell derived from the X-ray structure. The structure derived from this map shows both the out-of-plane carbon atoms, and the up and down position of the chlorines. The contrast varies because of the overlap of atoms within the molecule and from adjacent molecules. In this projection the molecule shows a 2-fold rotation axis, and because the chlorines project above and below the mean molecular plane, the conformation is uniquely established as D_{3d} .

Applicability

The molecule studied here was much more resistant to electron beam damage than most organic molecules. Proteins and aliphatic hydrocarbons are $\sim 10^2$ times weaker, but the attainable

resolution depends on the inverse root of the sustained dose. Cooling, and other techniques of protecting the specimen², can provide an additional factor of three, so that it is possible to obtain an image resolution below 1 nm even with the most delicate of specimens. Electron diffraction information is normally obtainable to 0.1 nm with aliphatic organic crystals, and better than 0.5 nm with proteins. Experimental difficulties in obtaining three-dimensional information remain, but methods are being developed to overcome these problems. The maximum entropy method, in the form described here, will routinely provide a structure to the resolution limit of the kinematic diffraction pattern, if initial low-resolution phase information is available. The calculations described here took about one hour of processor time on a Sun Sparcstation-2, so computing requirements should not hinder the use of the method. □

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Cloning and domain structure of the mammalian splicing factor U2AF

Phillip D. Zamore*, James G. Patton† & Michael R. Green‡

* Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, USA

† Laboratory of Molecular and Cellular Physiology, Harvard Medical School, Boston, Massachusetts 02115, USA

‡ Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01605, USA

A complementary DNA clone encoding the large subunit of the essential mammalian pre-messenger RNA splicing component U2 snRNP auxiliary factor (U2AF⁶⁵) has been isolated and expressed *in vitro*. It contains two functional domains: a sequence-specific RNA-binding region composed of three ribonucleoprotein-consensus sequence domains, and an arginine/serine-rich motif necessary for splicing but not for binding to pre-mRNA.

SPlicing of pre-mRNA *in vitro* involves the ordered assembly on the pre-mRNA of the spliceosome, a multicomponent complex containing small nuclear ribonucleoprotein particles (snRNPs) and proteins^{1–4}. Binding of U2 snRNP to the pre-mRNA branch site is the first of several steps in spliceosome assembly requiring ATP hydrolysis. U2 snRNP binding also requires U1 snRNP (ref. 5) and three protein factors: SF1, SF3 (ref. 6) and U2 auxiliary factor (U2AF)^{7,8}. U2AF purified from HeLa cells comprises a protein of relative molecular mass 65,000 (M_r 65K) (U2AF⁶⁵) and an associated 35K protein (U2AF³⁵)⁸. Of these two polypeptides, only U2AF⁶⁵ is required for *in vitro*

splicing of the two model pre-mRNA substrates tested⁹. U2AF⁶⁵ binds the polypyrimidine tract early during spliceosome assembly and in the absence of ATP.

Isolation of U2AF⁶⁵ cDNA

Figure 1a shows the U2AF⁶⁵ coding sequence and the partial sequence of 5' and 3' untranslated regions of the full-length cDNA clone. The full-length cDNA clone includes 5' untranslated sequences, the U2AF⁶⁵ coding region, about 800 nucleotides of 3' untranslated sequence and a 43-nucleotide poly(A)⁺ tail. The boxed sequences in the figure identify five proteolytic peptides determined by microsequencing. The U2AF⁶⁵ cDNA contains a 1,425-base-pair (475 amino acids) open reading frame predicted to encode a 53K protein. The DNA sequence upstream from the U2AF⁶⁵ coding region is G+C-rich and contains at least 13 CpG dinucleotides. Regions containing a high proportion of CpG dinucleotides are commonly associated with the 5' untranslated regions of genes¹⁰. These 'CpG islands' typically contain the sequence GGGCGG (G/C box). G/C boxes are present at nucleotides –110 and –97 in the putative 5' untranslated region of the U2AF⁶⁵ cDNA.

The deduced amino-acid sequence of U2AF⁶⁵ contains two classes of motifs found in other splicing factors: an arginine/serine-rich (RS) motif and three ribonucleoprotein consensus sequence (RNP-CS) domains. The RS-rich region,

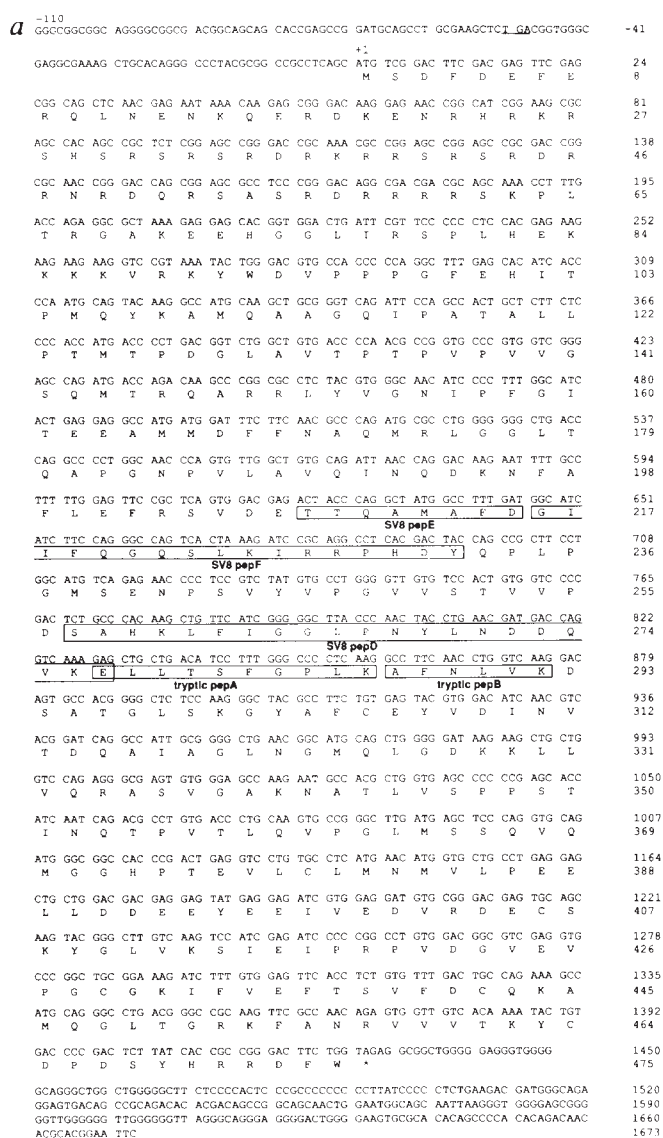


FIG. 1 Cloning of U2AF⁶⁵. **a**, Sequence of a U2AF⁶⁵ cDNA. The sequence of a U2AF⁶⁵ cDNA is shown, with the deduced U2AF⁶⁵ protein sequence indicated in one-letter amino acid code. Base pairs and amino acids are numbered separately. Peptides determined by microsequencing tryptic or *Staphylococcus aureus* V8 protease peptides are boxed. An in-frame termination codon upstream of the initiator methionine is underlined. Only sequences determined for both DNA strands are presented. **b**, Northern blot analysis. A ³²P-labelled DNA probe containing the U2AF⁶⁵ coding sequence was used to detect U2AF⁶⁵ mRNA in poly(A)⁺-enriched or total HeLa cell RNA by Northern blot analysis. RNA markers (kb) are on the left. The arrow indicates the major U2AF⁶⁵ mRNA. **c**, *In vitro* translation of U2AF⁶⁵ and three derivatives. Positions of *M_r* standards (K) are indicated on the left. The ³⁵S-labelled proteins were separated on a 12.5% SDS-polyacrylamide gel. **METHODS**. **a**, Purified HeLa cell U2AF (refs 8, 9) was separated by electrophoresis through an SDS-polyacrylamide gel and transferred to nitrocellulose. The portion of the membrane bearing the U2AF⁶⁵ polypeptide was digested with trypsin or *S. aureus* V8 protease (SV8)³⁷. Proteolytic peptides were resolved by HPLC and the sequences of five peptides determined by microsequencing. On the basis of the sequence of pepD, degenerate oligonucleotides were synthesized as primers in the polymerase chain reaction (PCR). The forward PCR primer was a 2:1 mixture of GC(N)CA(C/T)AA(A/G)CT(N)TT(C/T)AT and GC(N)CA(C/T)AA(A/G)TT(A/G)TT(C/T)AT; the reverse PCR primer was TC(C/T)TT(N)AC(C/T)TG(A/G)TC(A/G)TC. cDNA synthesis and PCR was according to a protocol supplied by Perkin-Elmer-Cetus. The PCR reaction contained 15 μM forward primers, 0.5 μM ³²P-end-labelled reverse primer, and 2.5 U *Taq* polymerase (Perkin-Elmer-Cetus). Amplification was for 30 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. The 59-nucleotide (nt) product was isolated from a

urea-polyacrylamide gel and chemically sequenced. A human fetal brain AGT10 library was then screened with the synthetic 54-nt oligonucleotide CAAG CTG TTC ATC GGG GGC TTA CCC AAC TAC CTG AAC GAC GAC CAG GTC AAG GA. Of ~3.6 × 10⁶ bacteriophage screened, two bacteriophage contained inserts that hybridized with the oligonucleotide probe in Southern blot analysis at 65 °C in 0.1 × SSC. The inserts from these AGT10 phage were subcloned and sequenced. The sequence of U2AF⁶⁵ will be deposited in the Genbank database. **b**, Total HeLa RNA was prepared and oligo(dT)-selected using standard methods. RNA was electrophoresed in a formaldehyde-agarose gel and transferred to a Nytran filter (Schleicher Schuell). Prehybridization and hybridization at 42 °C was in 5 × SSPE (0.9 M NaCl, 50 mM sodium phosphate, pH 7.4, 5 mM Na₂-EDTA), 0.5% SDS, 0.1% BSA, 0.1% Ficoll-400, 0.1% polyvinyl pyrrolidone, 10% (w/v) dextran sulphate and 50% (v/v) deionized formamide. The 1,649-bp *NotI*-*FspI* fragment of U2AF⁶⁵ was ³²P-radiolabelled to a specific activity of 2 × 10⁸ c.p.m. μg⁻¹ by random priming (Stratagene); 2 × 10⁵ c.p.m. ml⁻¹ was used in the hybridization. The final wash was with 0.1 × SSPE at 65 °C. **c**, The U2AF⁶⁵ coding sequence was fused to the 5' untranslated region of human β-globin downstream of an SP6 polymerase promoter in the vector pSP64 (Promega). To promote efficient *in vitro* translation, the second U2AF⁶⁵ amino acid, serine, was changed to alanine. RNA was transcribed with SP6 RNA polymerase (Promega Biotech) from plasmids linearized with *EcoRI* (full-length U2AF⁶⁵ and ΔRS), *PvuII* (bp 972; U2AF⁶⁵ ΔRNP3), or *StuI* (bp 683; U2AF⁶⁵ ΔRNP2, RNP3) and translated *in vitro* with wheat-germ extract (Ambion) supplemented with 100 μg ml⁻¹ rabbit liver transfer RNA (BRL). The derivative ΔRS, constructed by PCR and standard cloning techniques, contains a *Bam*HI restriction site in place of amino acids 25–63. For analysis of protein products, ³⁵S-methionine was included in the translation reaction.

TABLE 1 Binding of GST-U2AF Δ 1-63 to various pre-mRNA polypyrimidine tracts

RNA	Branch point/polypyrimidine tract sequence	K_d
B3P3	GGUA <u>AA</u> CUUUCU <u>UU</u> CUUCUCCUCCUGUCU UUCCUCUCUCUCU <u>UU</u> CCGC	$\sim 1 \times 10^{-8}$
B2P2	AGCCA <u>AA</u> CCUGGCACCCGUUGUGUGUCUC	$\sim 2 \times 10^{-6}$
MINX	UACUU <u>AA</u> CCUGUCCCUUUUUUCCACAG	$\sim 3 \times 10^{-7}$
β -globin	CACUG <u>AA</u> CUCUCUGCCU <u>UU</u> UGGUCU <u>UU</u> UCC ACCCUAG	$\sim 7 \times 10^{-7}$
PIP3	UACUU <u>AA</u> CCUGGGCCCUUUUUUCCACAG	$\sim 2 \times 10^{-6}$
PIP3Py4	UACUU <u>AA</u> CCUGGGCCCUUUUUUCCGAAUACAG	$> 2 \times 10^{-5}$
PIP3Py5	UACUU <u>AA</u> CCUGGGCCCUUUUUUCCGGAG	$\sim 2 \times 10^{-6}$
PIP3Py6	UACUU <u>AA</u> CCUGGGCCCU <u>UU</u> UUUCCACAG	$> 1 \times 10^{-5}$

The branch site and polypyrimidine tract sequence of the RNA substrates analysed are shown. Branch site adenosine nucleotides are underlined and bold; other underlined nucleotides represent point mutations or insertions. The rough apparent dissociation constant, K_d (M), of U2AF for each RNA is indicated. K_d was determined from the concentration of protein required to shift about 50% of the 32 P-labelled RNA into an RNA-protein complex. Because we have assumed both 100% activity for the recombinant protein and simple, non-cooperative binding, these data represent minimum estimates of the affinity of U2AF 65 for the various RNAs. Human β -globin RNA was generated from *Bam*I-linearized pSP64-H β miniE1 (ref. 31). MINX pre-mRNA was transcribed from a plasmid linearized with *Bam*HI (ref. 30). B3P3 and B2P2 RNAs are described in ref. 28; PIP3Py4, 5, 6 are described in ref. 24.

encompassing amino acids 25–63, is similar to the RS motifs of the U1 snRNP 70K protein $^{11-14}$, the splicing factor ASF/SF2 (refs 15, 16), and the *Drosophila* splicing regulators *transformer* 17 , *transformer-2* (refs 18, 19), *suppressor-of-sable* 20 and *suppressor-of-white-apricot* 21 . Among the RNP-CS domains of U2AF 65 , RNP-CS 1 (amino acids 151–229) and RNP-CS 2 (amino acids 261–334), which are the N-terminal and central RNP-CS domains, contain amino-acid sequences closely matching the consensus hexamer (or 'RNP2' element) and octamer (or 'RNP1' element) sequences characteristic of this RNA-binding protein domain. RNP-CS 3 (amino acids 393–462), the C-terminal domain, partially diverges from these consensus elements (see Fig. 4a for an alignment of the three RNP-CS domains) 22,23 . The U2AF 65 sequence contains no identifiable helicase or nucleotide-binding motifs.

To determine whether the cDNA we isolated contains most of the U2AF 65 mRNA, we did a northern blot. A hybridization probe containing the U2AF 65 coding region was used to detect U2AF 65 mRNA in HeLa cell poly(A) $^{+}$ -enriched and total RNA. Figure 1b shows that the cDNA hybridized with a roughly 2.4-kilobase (kb) mRNA. This 2.4-kb mRNA was not present in poly(A) $^{-}$ RNA (data not shown). Thus, the cDNA represents about 95% of U2AF 65 mRNA. An additional minor mRNA was also detected, which may result from alternative splicing.

To verify that the cDNA encodes full-length U2AF 65 protein, it was translated in a wheat-germ extract. Figure 1c shows the 35 S-labelled translation products separated on an SDS-polyacrylamide gel. Also shown are the *in vitro*-translated U2AF 65 derivatives used in the experiments described below. Like HeLa U2AF 65 , the U2AF 65 translation product migrated at an apparent $M_r \sim 65$ K, and it comigrated with purified HeLa U2AF 65 (data not shown), demonstrating that the cDNA encodes all of U2AF 65 .

To determine whether the cDNA encoded functional U2AF 65 , we used a previously described biochemical complementation assay 9 . U2AF is quantitatively depleted from HeLa cell nuclear extracts by chromatography on poly(U)-Sepharose in the presence of 1M KCl. The resulting U2AF-depleted nuclear extract (Δ U2AF-NE) cannot support pre-mRNA splicing of an adenovirus major late (Ad ML) pre-mRNA. Addition of purified HeLa U2AF to the Δ U2AF-NE restored its ability to splice. Figure 2 shows that U2AF 65 , transcribed and translated *in vitro*

from the cloned U2AF 65 cDNA (IVT U2AF), also restored splicing to the Δ U2AF-NE, whereas a translation reaction not programmed with mRNA (mock) did not (see also Fig. 4c). Thus, the cDNA encodes all the amino acids necessary to produce active U2AF 65 .

RS motif is essential

Although the RS motif is present in several metazoan splicing factors, whether it is essential for splicing or provides some other function (such as subcellular targeting) is unknown. We tested whether the RS motif was required for the *in vitro* activity of U2AF. Figure 2 shows that removal of the 39-amino-acid RS motif (amino acids 25–63) severely compromised the ability of U2AF 65 to support pre-mRNA splicing (IVT wild-type versus IVT Δ RS) and U2 snRNP binding (data not shown). Deletion of the RS motif did not prevent the protein from binding RNA (see below) or U2AF 35 (M. Zhang, P.D.Z. and M.R.G., manuscript in preparation). We conclude that the RS motif of U2AF 65 is directly involved in pre-mRNA splicing.

RNA binding

HeLa U2AF binds specifically to the polypyrimidine tract of pre-mRNAs 8 . The isolation of a U2AF 65 cDNA enabled us to investigate the specificity and mechanism of U2AF binding in greater detail. We constructed, overexpressed in *Escherichia coli*, and purified a series of glutathione-S-transferase (GST)/U2AF fusion proteins. The RNA-binding properties of the fusion proteins were tested using a mobility shift assay.

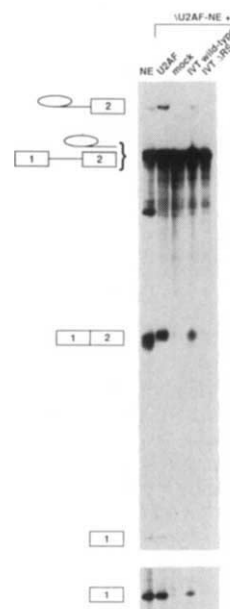


FIG. 2 *In vitro* translated U2AF 65 can substitute for HeLa U2AF in a splicing assay. Splicing of the MINX pre-mRNA was analysed using Δ U2AF-NE reconstituted with HeLa nuclear extract (NE), purified HeLa U2AF (HeLa U2AF), wheat-germ extract not programmed with RNA (mock), programmed with *in vitro*-translated U2AF 65 (IVT wild-type) or U2AF 65 lacking the RS motif (IVT Δ RS). Bottom panel shows a longer exposure of the region of the gel corresponding to free exon 1. The positions of splicing products are indicated diagrammatically at left. They correspond to (from top): lariat intermediate, lariat product, pre-mRNA, spliced mRNA, and free exon 1.

METHODS. *In vitro* splicing was in the presence of polyvinyl alcohol as in ref. 9. To a standard 25- μ l reaction, we added 14 μ l Δ U2AF-NE and 1 μ l of a wheat-germ translation reaction (done with 100 μ M methionine instead of 35 S-methionine as described in Fig. 1c). In lane 2, 1 μ l purified HeLa U2AF (refs 8, 9) was also added. Splicing reactions were done for 2 h, deproteinized, and then analysed on a 13% polyacrylamide gel containing 50% urea (w/v).

When full-length U2AF⁶⁵ was fused to GST (GST-U2AF), the protein bound tightly to RNAs containing a polypyrimidine tract. Figure 3a shows that GST-U2AF bound to an Ad ML-derived pre-mRNA (PIP3)²⁴. By contrast, GST-U2AF bound ~25-fold less well to a pre-mRNA lacking the polypyrimidine tract/3' splice site (PIP3ΔPyAG). When tested for binding using this mobility shift assay, purified HeLa U2AF bound with affinity and specificity comparable to GST-U2AF (data not shown).

Figure 2 shows that the RS motif is essential for U2AF⁶⁵ activity. One possible role for the RS region is in binding to the pre-mRNA: several sequence-specific RNA-binding proteins

contain an arginine-rich binding motif (reviewed in ref. 25). To address this issue, we constructed a GST fusion protein (GST-U2AFΔ1-63) that contains the three U2AF⁶⁵ RNP-CS domains (amino acids 64-475) but lacks the RS region. This protein bound to polypyrimidine-tract containing RNAs, PIP3 and a 5' splice site deletion of this RNA, PIP3Δ5'ss, with comparable affinity to that of full-length U2AF⁶⁵, but did not bind to the RNA lacking the polypyrimidine tract, PIP3ΔPyAG (Fig. 3b). Therefore, the RS motif is dispensable for U2AF⁶⁵ sequence-specific RNA binding. We conclude that the U2AF⁶⁵ sequence-specific RNA-binding domain must be in the C terminus of the protein, which consists of three RNP-CS domains. The RS domain must therefore have a role in U2AF activity distinct from RNA binding.

RNP-CS domains mediate both sequence-specific RNA binding and protein-protein interactions²⁶. We therefore asked whether all three RNP-CS domains were required for sequence-specific RNA binding. We constructed a series of GST-fusion proteins containing one or more of the U2AF⁶⁵ RNP-CS domains. The three U2AF⁶⁵ RNP-CS domains are shown aligned to each other in Fig. 4a. We determined the affinity of each derivative relative to that of GST-U2AFΔ1-63 (labelled RNP-CS 1, 2, 3 in Fig. 4b), which contains the three RNP-CS domains. Figure 4b shows that all three RNP-CS domains are important for high-affinity binding to three different RNAs containing polypyrimidine tracts. The sequences of these RNAs appear in Table 1. Deletion of RNP-CS 3 (amino acids 325-475; labelled RNP-CS 1, 2 in figure), reduces the affinity of U2AF⁶⁵ 9-fold for PIP3, 12-fold for MINX, and more than 350-fold for the α -tropomyosin B3P3 RNA. Deletion of both RNP-CS 3 and RNP-CS 2 (amino acids 229-475) slightly reduces further the affinity of the protein (RNP-CS 1 in figure). A protein lacking the N-terminal RNP-CS 1 (amino acids 1-228) but containing RNP-CS 2 and RNP-CS 3 (labelled RNP-CS 2, 3 in figure) binds 100- to nearly 5,000-fold less well than the intact protein. These results suggest that all three RNP-CS domains contribute to high-affinity RNA-binding. Significantly, all of these U2AF⁶⁵ derivatives discriminate between RNAs containing or lacking the polypyrimidine tract (data not shown).

Efficient pre-mRNA splicing requires a polypyrimidine tract, the binding site of U2AF⁶⁵. To confirm that U2AF⁶⁵ function requires an intact sequence-specific RNA-binding region, we analysed the ability of U2AF⁶⁵ derivatives to support splicing. These *in vitro* translation products are shown in Fig. 1c and the splicing assays in Fig. 4c. Deletion of RNP-CS 3 (amino acids 325-475) reduced by 12 times the affinity of U2AF⁶⁵ for the Ad ML MINX pre-mRNA (see Fig. 4b) and decreased correspondingly the U2AF⁶⁵ splicing activity (IVT- Δ RNP3). When both RNP-CS 2 and RNP-CS 3 (amino acids 229-475) were removed, splicing was undetectable (IVT- Δ RNP2, RNP 3). We conclude that an intact sequence-specific RNA-binding domain, in addition to the RS region, is essential for U2AF⁶⁵ activity.

Although the polypyrimidine tract is a consensus splicing element, there is a remarkable diversity of polypyrimidine tract sequences among mammalian pre-mRNAs. The polypyrimidine tract sequence affects splice site choice and spliceosome assembly²⁷⁻²⁹. Because U2AF binding is an early step in spliceosome assembly, polypyrimidine tract sequence seemed likely to affect U2AF⁶⁵ binding. We analysed GST-U2AFΔ1-63 binding for eight different pre-mRNA polypyrimidine tracts: two rat α -tropomyosin RNAs, B3P3 (the default intron) and B2P2 (the intron specific for smooth muscle)²⁸; two different Ad ML intron 1 derivatives, MINX³⁰ and PIP3²⁴; human β -globin intron 1 (ref. 24); and three PIP3 point mutants. Table 1 summarizes the sequences of these RNAs and the affinity of GST-U2AFΔ1-63 for each.

GST-U2AFΔ1-63 bound these RNAs with remarkably different affinities, spanning a 200-fold range. Qualitatively, GST-U2AFΔ1-63 bound B3P3 $\gg$ MINX $>$ human β -globin $>$ PIP3 $\sim$ B2P2. A comparison of these sequences reveals that

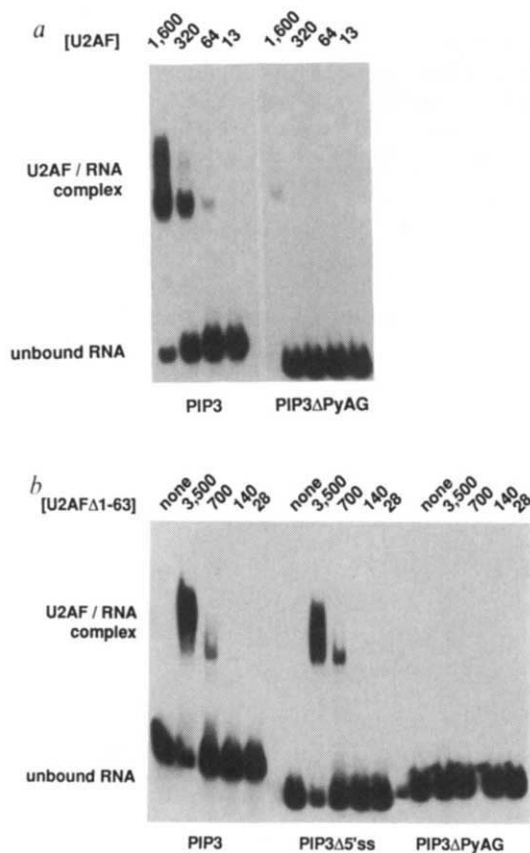


FIG. 3 Sequence-specific binding of U2AF⁶⁵ requires the RNP-CS domains but not the RS motif. *a*, Binding of GST-U2AF. Various amounts of GST-U2AF were incubated with ³²P-labelled RNA for 1 h at 25 °C and the complexes were separated by electrophoresis on a native polyacrylamide gel. The RNA substrate is indicated below each panel and the concentration of fusion protein (nM) is indicated above each panel. *b*, Binding of GST-U2AFΔ1-63 to wild-type and mutant polypyrimidine tracts.

METHODS. GST-U2AF fusion proteins were expressed from U2AF⁶⁵ derivatives in the pGEX series of vectors³⁸. GST-U2AF contained U2AF⁶⁵ amino acids 1-475 in pGEX-3X; GST-U2AFΔ1-63, amino acids 64-475 in pGEX-2T. Fusion proteins were overexpressed and purified according to ref. 39. GST-U2AF and GST-U2AFΔ1-63 were further purified on poly(U)-Sephacrose⁸. Protein concentration was estimated by comparing the intensity of the full-length fusion protein with a BSA standard (Pierce) on an SDS-polyacrylamide gel stained with Coomassie blue R-250. RNA-binding reactions contained 20 U RNasin (Promega Biotech) and 1 mg ml⁻¹ yeast tRNA in 50 mM KCl, 10 mM HEPES-KOH (pH 8.0), 0.025% Nonidet P-40, 1 mM dithiothreitol, and 10% glycerol. The ³²P-labelled RNA concentration was ~10⁻¹⁰ M. RNA-protein complexes were analysed on 4% polyacrylamide gels (37.5:1, acrylamide:bis-acrylamide) containing 1% glycerol and 0.5× TBE. Gels (pre-run for 1 h) were electrophoresed at a constant power of 3.5 W at 4 °C. Uncapped RNAs were transcribed from linearized plasmids using SP6 or T7 RNA polymerase. Plasmid PIP3 used in this study is the same as that described in ref. 24 except that adenosine 117 was deleted. PIP3ΔPyAG was constructed by deletion of nucleotides 137-166 from PIP3; PIP3Δ5'ss was constructed by deletion of nucleotides 11-64 from PIP3. For transcription, these plasmids were linearized with *Hind*III.

U2AF⁶⁵ binds RNA more tightly as pyrimidine content and polypyrimidine tract length increase.

Table 1 also shows that subtle changes in the polypyrimidine tract can also have an effect on U2AF⁶⁵ binding. The binding of U2AF⁶⁵ to the three point mutants of the Ad ML PIP3 pre-mRNA emphasizes this phenomenon. PIP3Py4 contains a single U→A change in the polypyrimidine tract and several nucleotide insertions between the polypyrimidine tract and the 3' splice site; U2AF⁶⁵ bound PIP3Py4 10-fold less well than wild-type PIP3. In PIP3Py6, there are three U→A changes in the polypyrimidine tract. U2AF⁶⁵ bound this RNA about five-fold less well than wild-type PIP3. By contrast, PIP3Py5, which has a wild-type polypyrimidine tract but contains an AC→GG change at the 3' splice site, bound U2AF⁶⁵ as well as wild-type PIP3.

U2AF⁶⁵ modular structure

The sequence of U2AF⁶⁵ contains four separate sequence motifs: an RS region and three RNP-CS-type RNA-binding modules. Our results demonstrate that each of these domains has a role in U2AF function (see Fig. 5).

The first functional domain of U2AF⁶⁵ is the N-terminal RS motif. We have shown that this domain, which is similar to those in other metazoan splicing factors, is necessary for efficient U2AF function but dispensable for RNA binding. We therefore refer to the RS motif as the U2AF⁶⁵ effector domain.

The main function of the RS motif has been proposed to

target proteins to a particular subnuclear compartment³². Although not inconsistent with the RS motif playing a part in subcellular targeting, our data indicate that the U2AF⁶⁵ RS motif has an intrinsic role in spliceosome assembly and splicing. We note that the intranuclear distribution of U2AF (refs 9, 33) differs from that of the RS proteins studied by Bingham and colleagues³².

An important question raised by our studies is: how does the U2AF⁶⁵ RS motif promote binding of U2 snRNP to the pre-mRNA branch site? The RS motif might contact a specific protein, such as a U2 snRNP structural polypeptide. Alternatively, the U2AF⁶⁵ RS motif might contact RNA. One attractive idea is that U2AF⁶⁵ bound at the polypyrimidine tract could extend its RS region upstream and stabilize the base-pairing interaction between U2 snRNA and the pre-mRNA branch site. This hypothesis would help to explain the observation that, in mammalian cells, RNA branches almost always form near the polypyrimidine tract/3' splice site¹.

The second functional domain of U2AF⁶⁵ is a sequence-specific RNA-binding region composed of three RNP-CS domains. Each of the three U2AF⁶⁵ RNP-CS domains binds preferentially to pyrimidine-rich sequences, but no individual RNP-CS has the high specificity and affinity of intact U2AF⁶⁵.

U2AF and splice site choice

The U2AF⁶⁵ binding data help explain features of constitutive and regulated splicing. Previous studies have shown that the

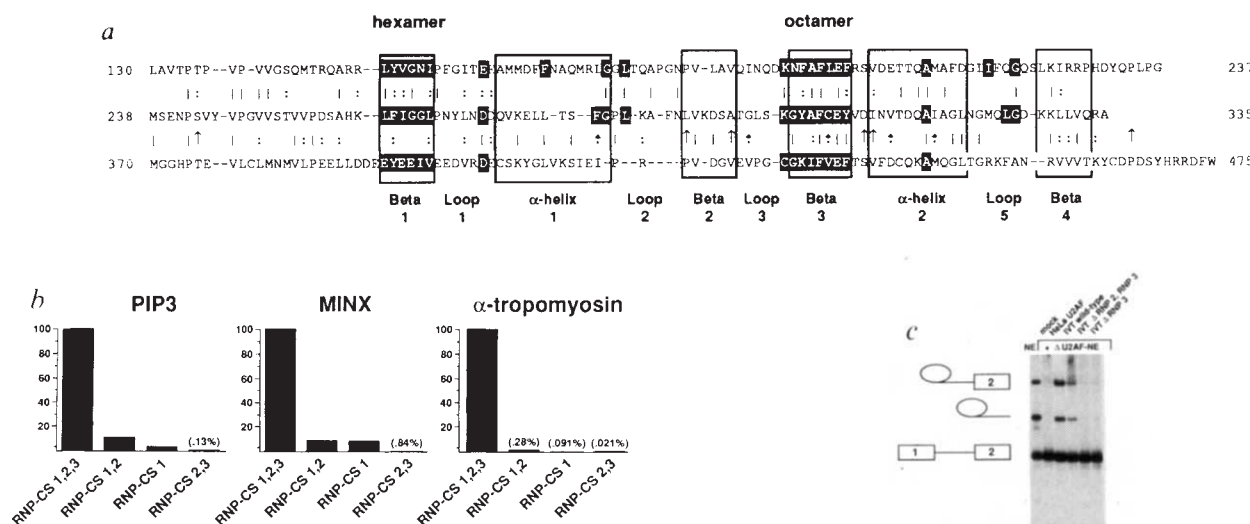
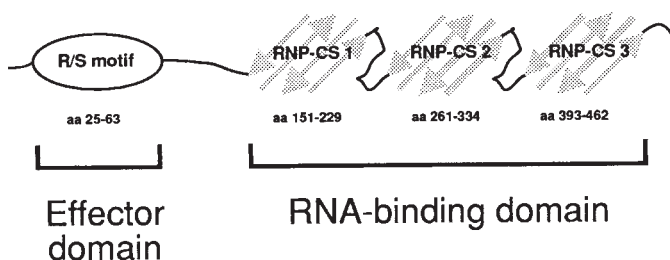


FIG. 4 All three RNP-CS domains contribute to sequence-specific RNA binding. *a*, Alignment of the three U2AF RNP-CS domains. Amino acids conserved among RNP-CS domains of different proteins are displayed as white letters in a black box; the conserved hexamer and octamer sequences are labelled at the top. Protein structural features of the RNP-CS domain as defined in ref. 23 are boxed and labelled at the bottom. Symbols between the sequences indicate amino-acid identity (|) or similarity (:) among U2AF⁶⁵ RNP-CS domains. An arrow on these symbols indicates identity or similarity (dotted arrow) between RNP-CS 3 and RNP-CS 1 but not RNP-CS 3 and RNP-CS 2. *b*, Binding of U2AF RNP-CS derivatives relative to the entire sequence-specific RNA-binding domain for the PIP3, MINX, and B3P3 RNAs. The binding of the U2AF derivatives containing RNP-CS 1 and 2 (RNP-CS 1, 2), RNP-CS 1, or RNP-CS 2 and 3 (RNP-CS 2, 3) to the Ad ML-derived RNAs PIP3 and MINX, and the α -tropomyosin B3P3 RNA was determined. For each RNA, the binding affinity of the U2AF derivatives is reported relative to the binding of the fusion protein GST-U2AF Δ 1-63 (labelled RNP-CS 1, 2, 3), which contains the three intact RNP-CS domains. *c*, The U2AF⁶⁵ RNP-CS domains are needed for U2AF⁶⁵ to function in pre-mRNA splicing. NE, HeLa nuclear extract. U2AF-depleted HeLa nuclear extract (Δ U2AF-NE) was rescued with wheat-germ extract not programmed with RNA (mock), with purified HeLa U2AF (HeLa U2AF), or with *in vitro* translated U2AF⁶⁵ (IVT wild-type), U2AF⁶⁵ lacking RNP-CS 2 and RNP-CS 3 (IVT Δ RNP2, RNP3), or U2AF-lacking RNP-CS 3 (IVT

Δ RNP3). Bottom panel shows a longer exposure of the region of the gel corresponding to free exon 1.

METHODS. MacVector computer software (version 3.5; IBI) was used for sequence analysis. Fusion proteins were purified and RNA binding determined as for Fig. 3. GST-RNP1, 2 corresponds to U2AF⁶⁵ amino acids 64-324 in pGEX-2T; GST-RNP1, amino acids 64-228 in pGEX-2T; GST-RNP2, 3, amino acids 230-475 in pGEX-3X. *In vitro* splicing was as for Fig. 2.

FIG. 5 Functional organization of U2AF⁶⁵.

length and pyrimidine content of the polypyrimidine tract has a profound effect on splicing²⁷⁻²⁹ and it seems likely that U2AF⁶⁵ is at least one factor that mediates these effects. For the alternatively spliced α -tropomyosin pre-mRNA, U2AF⁶⁵ bound the B3P3 polypyrimidine tract (default intron) ~200-fold better than

B2P2 polypyrimidine tract (regulated intron). Thus, the relative affinities of U2AF⁶⁵ for these two sequences can explain the default splicing pattern of this pre-mRNA (reviewed in ref. 34).

Exclusion of U2AF from a strong polypyrimidine tract or recruitment of U2AF to a weak sequence may regulate alternatively spliced pre-mRNAs, as in the control of 3' splice site selection during *Drosophila* sex determination (reviewed in ref. 35). In an *in vitro* system, the *Drosophila* regulatory protein Sex-lethal can repress binding of U2AF to the strong (default) polypyrimidine tract of the *Drosophila transformer* (*tra*) pre-mRNA thereby allowing use of the weak (regulated) site (J. Valcarcel, P.D.Z. and M.R.G., manuscript in preparation). Conversely, for the *doublesex* pre-mRNA, recruitment of U2AF by the *tra* and *transformer-2* gene products has been proposed to explain the alternative splicing pattern³⁶. The availability of a U2AF clone now provides a means to examine in greater detail the mechanism of this constitutive splicing factor and its role in alternative splicing. □

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LETTERS TO NATURE

A 6.5-day periodicity in the recurrent nova V404 Cygni implying the presence of a black hole

J. Casares*, P. A. Charles†‡ & T. Naylor§||

* Instituto de Astrofísica de Canarias, 38200 La Laguna, Tenerife, Spain

† Royal Greenwich Observatory, Apartado 321, 38780 Santa Cruz de La Palma, Tenerife, Canary Islands

‡ Department of Astrophysics, Nuclear Physics Building, Keble Road, Oxford OX1 3RH, UK

§ Institute of Astronomy, Madingley Road, Cambridge CB3 0HA, UK

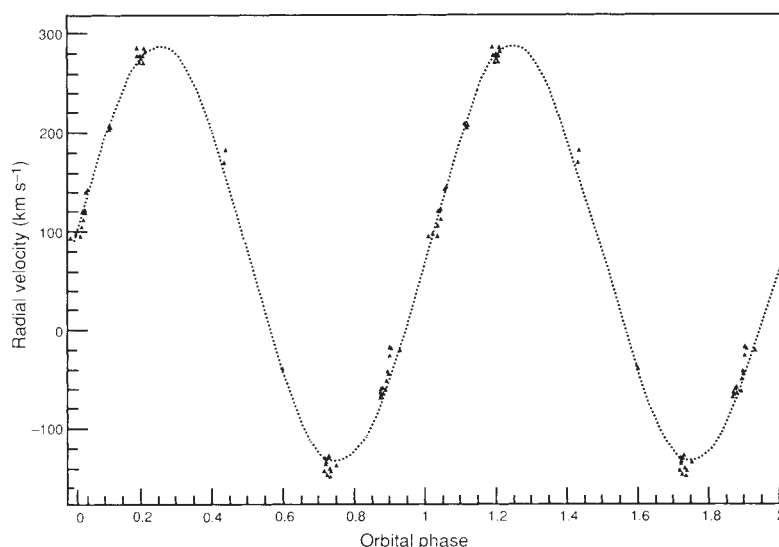
THE X-ray transient source GS2023+338 was discovered in outburst by the Ginga satellite in 1989 (ref. 1) and has since been identified with the previously known recurrent nova V404 Cygni². This system is recognized to be a low-mass X-ray binary³, with X-ray behaviour similar to black hole systems⁴, but attempts to deduce an orbital period from photometry⁵⁻⁹ and spectroscopy^{10,11} have yielded modulations with periods from 10 minutes to 6 hours. Two years after the outburst, we have used the William Herschel

Telescope to find absorption features in V404 Cyg characteristic of a late G or early K star with a radial velocity curve of amplitude $211 \pm 4 \text{ km s}^{-1}$ and period 6.473 ± 0.001 days. The deduced mass function of $6.26 \pm 0.31 M_{\odot}$ is a firm lower limit to the mass of the compact object, which for reasonable assumptions of orbital inclination and companion star mass must be a black hole with probable mass in the range 8–15.5 $M_{\odot}$. We consider this the most persuasive case yet for the existence of a black hole.

Unequivocal evidence for a stellar-size black hole in the Galaxy has been sought for over two decades¹². CygX-1 is a strong candidate, but the massive (normal) companion star makes it difficult to set a lower limit of better than 3 $M_{\odot}$ to the mass of the compact object. The soft-X-ray transients have yielded potentially more important candidates because of the low mass of the companion (or secondary) star and its faintness after the X-ray outburst has subsided into quiescence. The 1975 transient A0620-00 is currently considered to be the strongest black-hole candidate¹³, with a mass function of 2.9 $M_{\odot}$, which represents the lower limit to the mass of the compact object. The discovery of a new X-ray transient prompts a rapid search for an optical counterpart in order to determine whether the companion star is of low mass (as indicated by the change in brightness from quiescence to outburst). If so, the object is marked for closer scrutiny when it has returned to quiescence and the companion star can be studied without being masked by the high luminosity of the accretion disc. Although X-ray and optical light curves similar to those of A0620-00 are

|| Present address: Department of Physics, Keele University, Keele, Staffordshire ST5 5BG, UK.

FIG. 1 Radial velocity curve and best sine fit for the secondary of V404 Cyg with respect to the rest frame of 61 Cyg A (the template used in the initial cross-correlation analysis), which has a gamma velocity of $-64.5 \pm 2.2 \text{ km s}^{-1}$. The ephemerides given in the text have been corrected for this.



considered sufficient to label an object a 'black-hole candidate'¹⁴, the only certain way of identifying the compact object is to study the radial velocity of the secondary star and measure the mass function.

The 1989 outburst of GS2023+338 was remarkable for its rapid and erratic variability, and for its very high X-ray luminosity at maximum ($\sim 1.5 \times 10^{38} (d/1 \text{ kpc}) \text{ erg s}^{-1}$)⁴. From our estimate¹⁰ of the minimum distance to V404 Cyg of 1.5–2 kpc, this implies an X-ray luminosity far in excess of the Eddington limit for a $1 M_{\odot}$ object. During the decline from outburst, several photometric periodicities were reported^{5,8,9}, but such modulations during outburst and decline are common amongst X-ray transients (for example, Aql X-1, 1.3 d (ref. 15) A0620–00, 7.8 d (ref. 16)) and usually are subsequently found to be unrelated to the true orbital period. Their origin remains unknown. Therefore, we shall only concern ourselves here with spectroscopic modulations detected during quiescence (1 year after outburst or more).

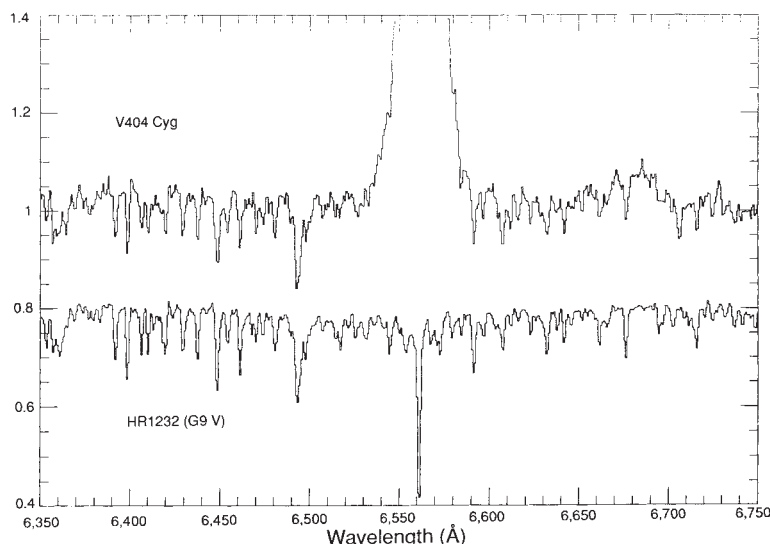
We obtained 55 red spectra (wavelength 6,050–6,990 Å, 0.74 Å pixel⁻¹ dispersion) of V404 Cyg at the Observatorio del Roque de los Muchachos using the 4.2-m William Herschel Telescope (WHT), equipped with the ISIS triple spectrograph, on the nights of 6 and 7 July, and 6–9, 17–20 and 22 August 1991, covering intervals of between 0.5 and 6 hours per night. Seeing was excellent (always $\leq 1''$) and a 0.6–0.8-arcsec slit was used. Each set of three 1,200-s exposures was bracketed by a Cu–Ne

arc for wavelength calibration, the stability of which was verified using the OH sky lines (at 6,300.2, 6,562.8 and 6,863.9 Å) to be within 0.2 Å. We also included 18 spectra¹¹ from 22 July 1990 obtained on the 2.5-m Isaac Newton Telescope, to give a total of 73 spectra for analysis.

Even in individual WHT spectra we could detect the 6,495-Å absorption feature (a blend of mainly Ca I and Fe I; ref. 17), which is a hallmark of late G and K type stars. Radial velocities were derived from this feature by cross-correlating the spectra in the 6,350–6,530 Å range with the K5 template star 61 Cyg A (refs 17, 18). These clearly demonstrated a large-amplitude, smooth modulation with a period of ~ 6.5 days (Fig. 1). To be certain that this was the true period and not an alias (caused by the temporal spacing of our observations), we did a periodogram analysis and examined other 'peaks' in the power spectrum. The next best periods are at 0.87 and 1.17 days, but both of these give very poor radial velocity curves when folded (as shown by the change in χ^2_{ν} from 1.2 for the 6.5-day period to 14.3 for the next best period, the formal probability for which is vanishingly small ($< 10^{-40}$) for our 52 degrees of freedom). Finally, we used the one-dimensional CLEAN deconvolution algorithm¹⁹ which effectively corrects the periodogram for the uneven spacing of the data. This confirmed the above analysis as it yielded a δ -function at a frequency corresponding to 6.47 days. All other peaks were indeed aliases.

The radial velocity curve of the secondary shown in Fig. 1

FIG. 2 Summed spectrum of V404 Cyg after Doppler correction into the rest frame of the secondary. A spectrum of a G9 V star is also plotted for comparison.



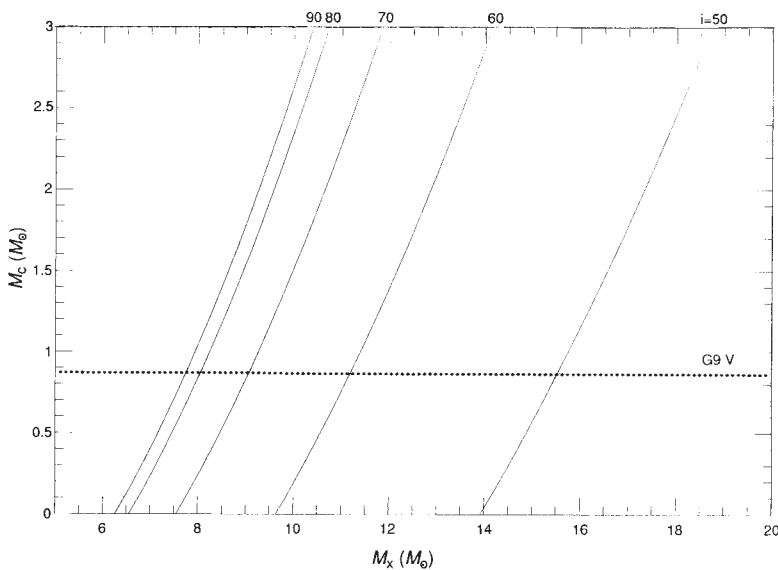


FIG. 3 The mass function of V404 Cyg strongly supports the hypothesis of the compact object being a black hole. The horizontal dotted line represents the mass of a G9 V secondary. Inclination constraints ($80^\circ \geq i \geq 50^\circ$) suggest masses for the compact object in the range 8–15.5 $M_\odot$.

was actually obtained by cross-correlation with the Doppler-shifted summed spectrum of V404 Cyg itself. A sine fit to Fig. 1 gives the following orbital parameters (with $\pm 1\sigma$ errors): $P = 6.47259 \pm 0.0009$ d; $\gamma = 11.4 \pm 6$ km s $^{-1}$; $K = 210.6 \pm 4$ km s $^{-1}$; $T_0 = 2,448,477.353 \pm 0.025$ d, where T_0 corresponds to absolute phase 0.0 (inferior conjunction of the secondary star). Doppler correction to the rest frame of the secondary using this fit causes the late-type features to emerge very clearly in the summed spectrum (Fig. 2), providing further confirmation of the correctness of the 6.5-day period. To estimate the spectral type more accurately we obtained spectra of bright stars (luminosity classes III and V) in the range G4–K7, which show that the secondary is either a G9(± 1)V or K0(± 4)III star. To illustrate this, a G9 V spectrum is also plotted in Fig. 2 with an appropriate offset. Such a large velocity amplitude is remarkable for this period, and the mass function is

$$f(M) = \frac{(M_x \sin i)^3}{(M_x + M_c)^2} = 6.26 \pm 0.31 M_\odot.$$

A lower limit to M_x (with $i = 90^\circ$ and the companion mass $M_c = 0$) is $6.26 M_\odot$, which substantially exceeds the $3 M_\odot$ maximum allowed mass of a neutron star^{12,13} and we conclude that the compact object must be a black hole. Figure 3 shows the dependence of M_x on M_c and i , where the horizontal dotted line represents a G9 V secondary. As in the case of A0620–00, M_x depends weakly on M_c . No X-ray eclipses were observed⁴ during the outburst, implying $i \leq 80^\circ$ (for a Roche-lobe filling secondary) and further constraining M_x . On the other hand the double-peaked H α profiles¹¹ suggest $i \geq 50^\circ$. An additional constraint could come from the detection of the compact object's K velocity.

Recent work on the structure of high-density baryonic matter has led to equations of state that are very different from ordinary nuclei²⁰, allowing the possibility of neutron star masses outside previously determined ranges. Such 'Q-stars' would be an alternative to a black hole in the present case. But all accurately determined neutron star masses so far^{21,22} occupy a narrow range of masses below $2 M_\odot$. Our observations demonstrate that the V404 Cyg compact object must show more exotic states of matter than 'conventional' neutron stars. Heavy ($> 2 M_\odot$) neutron stars X-ray pulsars) have not yet been found, nor do the best black-hole candidates (V404 Cyg and A0620–00) show characteristics of neutron stars (X-ray pulsations or bursts). If these characteristics are suppressed in Q-stars (for example, by a low magnetic field) then differentiating observationally between a black hole and a Q-star may prove extremely difficult.

The most straightforward model for V404 Cyg is a binary system consisting of a black hole and a K0 III secondary. A main-sequence star is ruled out because its radius would only be 0.14 of its Roche-lobe radius, and it would thus be unable to transfer mass to the compact object. It must be close to the Roche-lobe radius to account for the X-ray outbursts. Such a giant system would be similar to the low-mass X-ray binary Cyg X-2 (ref. 23), which consists of an F2 III + $1.5 M_\odot$ neutron star in a 9.8-day binary at a distance²⁴ of ~ 8 kpc, except that Cyg X-2 is not a transient source. The problem with this hypothesis is that the visual luminosity of a G9 or K0 III star would place V404 Cyg at ~ 11 kpc (assuming $A_v = 3$; see ref. 10) in order to match the current m_v of ~ 19 (in fact this is a lower limit, as we have not taken into account any contribution from the accretion disk). Also, if V404 Cyg were at such a large distance, its peak X-ray luminosity in outburst would have been a spectacular 1.5×10^{40} erg s $^{-1}$.

Limits on the giant hypothesis can be set by considering the rotational velocity of such a star co-rotating with its massive companion in 6.5 days. The Roche-lobe radius of the secondary star can be calculated from $R_c = 0.234 P^{2/3} M_c^{1/3} R_\odot$ (where P is in hours), which is derived from Kepler's third law, and the expression relating Roche-lobe radius with mass ratio²⁵. A K0 III would have a mass of $4 M_\odot$ (ref. 26) and hence $R_c = 10.7 R_\odot$, which is smaller than its nominal radius (in which case the system must have evolved through mass transfer, as expected). The co-rotational velocity is then 85 km s $^{-1}$. Our spectra (Fig. 2) will only allow a maximum rotational velocity of 55 km s $^{-1}$ (calculated by broadening the template star spectrum and comparing with V404 Cyg) which would imply an inclination $< 40^\circ$. As we require an inclination of at least 50° to be compatible with the H α profiles, our upper limit to the observed rotational velocity corresponds to $v_{\text{rot}} \leq 72$ km s $^{-1}$, giving $M_c \leq 2.5 M_\odot$ and $R_c \leq 9 R_\odot$. Observations at higher spectral resolution will be required to refine this limit.

Alternatively, V404 Cyg may be a triple system, in which the G/K star observed in our spectra is a main sequence star orbiting a black hole/late-type-dwarf binary. The late-type dwarf must be $\leq 0.5 M_\odot$ for its luminosity to be much lower than that of the G/K star, and thus not be detected in our spectra. This inner binary would then be similar to other X-ray transients, but the third star allows us to place a limit on its total mass of $6.26 M_\odot$. Because the late-type star cannot be more than $\sim 0.5 M_\odot$, the compact object must still be a black hole, as discussed above. Such a model has the further attraction that the 6-h spectroscopic modulation in H α (ref. 11), which a preliminary analysis shows is also present in our data, may correspond to the orbital period

of the inner pair. We note that the inner binary ($P = 5.8$ hours, $M_{11} + M_{12} = 6.3 M_{\odot}$) would have a separation of $3.0 R_{\odot}$, and the outer binary ($P = 6.5$ days, $M_1 + M_2 = 7.1 M_{\odot}$) a separation of $28.2 R_{\odot}$. The ratio of these semimajor axes is >9 and hence the orbits are dynamically stable²⁷. No triple system has yet been confirmed in a low-mass X-ray binary, but 4U2129+47

(V1727 Cyg)^{28,29} is the best candidate, with a similar proposal being made for 4U1813-14 (GX17+2)³⁰. The question of how such a triple could have evolved to its present state in V404 Cyg is perplexing. Further observations of the H α emission of V404 Cyg might reveal modulations of the γ -velocity on the 6.5-day period and verify the existence of the triple system. $\square$

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A model for the radio brightness of the supernova remnant 1987A

Roger A. Chevalier

Department of Astronomy, University of Virginia, PO Box 3818, Charlottesville, Virginia 22903, USA

THE recently observed rise¹ in radio flux from SN1987A is earlier than predicted in models of the interaction with the observed line-emitting gas^{2,3}. The observed size of the radio source is roughly consistent with the expected position of the supernova shock wave, which is inside the optically emitting ring. The size is also consistent with the position of the termination shock of the blue supergiant progenitor wind, and the radio increase is attributed to the interaction of the supernova shock with this density jump. The required efficiency of electron acceleration and magnetic field amplification is larger than usually occurs in supernovae and supernova remnants. Possible reasons for the enhanced radio emission are particle acceleration by the weak reflected shock that travels through the interaction region, and the action of the Richtmyer-Meshkov instability near the initial termination-shock density jump. The action of these mechanisms is time-limited; the prediction of this model is that the radio flux should begin to decline within the next year.

The early radio emission observed⁴ from SN1987A can be attributed to the interaction of the supernova shock with the wind from the blue supergiant progenitor star^{2,5,6}. I take the stellar wind to be characterized by a mass-loss rate $\dot{M}_b = 6 \times 10^{-6} M_{\odot} \text{ yr}^{-1}$ and a velocity of $v_b = 550 \text{ km s}^{-1}$ (refs 2, 5), and the outer supernova density profile to be characterized by a power-law fit to a numerical model by Arnett^{2,7}. The interaction is then given by a self-similar solution⁸ and the outer shock radius is $R_{sh} = 7.5 \times 10^{16} t_{yr}^{0.87} \text{ cm}$, where t_{yr} is the time since the explosion in years. In June and July 1991, the observed angular size of the radio source¹ was $(1.0 \pm 0.1) \times (0.9 \pm 0.1) \text{ arcsec}$, corresponding to $(7.5 \pm 0.7) \times 10^{17} \times (6.7 \pm 0.7) \times 10^{17} \text{ cm}$ at a distance of 50 kpc. The expected diameter of the shocked region in the blue supergiant wind is $5.4 \times 10^{17} \text{ cm}$. As described below, the actual shock radius at this time may be smaller because of interaction with an enhanced density, but the overall agreement seems to be adequate; the value of $\dot{M}_b$ may have been overestimated and the shock radius is $\propto \dot{M}_b^{-0.13}$. In any case, the

diameter is less than that of the line-emitting ring, which is $1.3 \times 10^{18} \text{ cm}$ (ref. 9).

The early radio data, particularly that at 843 MHz, can be roughly fitted to a power-law decline $t^{-1.35}$ in the optically thin phase^{2,4}. This decline is consistent with the circumstellar interaction model and is expected to continue while the supernova shock is within the blue supergiant wind. Extrapolation of this evolution to November 1990 implies an 843-MHz flux of 0.08 mJy, whereas the observed flux was 7 mJy and rising¹. Here I attribute the rise to interaction with the termination shock of the blue supergiant stellar wind, where it is slowed by the swept-up red supergiant wind. The position of the shock depends on the properties of both the blue supergiant wind and the swept-up shell of red supergiant wind. The pressure in the shocked bubble is expected to be roughly uniform³, and the estimate from spherically symmetric theory¹⁰

$$p = \frac{3}{4} \left(\frac{16}{15} \right)^{2.5} \frac{\dot{M}_b v_b}{4\pi r_t^2}$$

where r_t is the termination shock radius, should apply. If the shocked wind fills most of the bubble volume, another estimate of the interior pressure is $p = 2\alpha E_i / 3V$, where E_i is the total energy deposited by the blue supergiant wind, α is the fraction of that energy that goes into internal energy in the bubble, and V is the volume of the bubble. I note that $E_i = \frac{1}{2} \dot{M}_b v_b^2 \tau$, where τ is the age of the fast wind, and that $\alpha = 2/3$ for interaction with a slow wind. Equating these pressure estimates yields

$$r_t = \left(0.32 \frac{V}{v_b \tau} \right)^{1/2}$$

Observations of the bipolar nebula¹¹ and a model fit to the observations³ imply $V = 1 \times 10^{55} \text{ cm}^3$. The ring around the supernova is expanding at 10.3 km s^{-1} (ref. 12), which, combined with the ring radius, yields an age of $2 \times 10^4 \text{ yr}$. The resulting shock radius is $r_t = 3 \times 10^{17} \text{ cm}$.

Although the radio emission was first observed¹ in July 1990, adjusting to the longer light-travel time to the supernova position gives an interaction time in November 1990. With the model for interaction with the blue supergiant wind given above, the outer shock radius is then $2.4 \times 10^{17} \text{ cm}$, the shock velocity is $1.7 \times 10^4 \text{ km s}^{-1}$, and the preshock density is $9.9 \times 10^{-24} \text{ g cm}^{-3}$. The supernova shock front is sufficiently close to the wind

termination shock for interaction in November 1990 to be plausible.

When the strong supernova shock wave hits the density discontinuity (a factor of 4) at the wind termination shock, a reflected shock is generated by an increase in pressure (by a factor of 3) at the discontinuity¹³. The reflected shock has a Mach number of 1.6, corresponding to a density jump by a factor of 1.86. The compression of the shock interaction region can increase the flux by compressing the magnetic field and the associated betatron acceleration, but this gives an increase of only a factor of 3–5, depending on the magnetic field geometry. If it is assumed that the magnetic energy density and the relativistic energy density are a constant fraction, f , of the thermal energy density, as is assumed in the model fit to the early radio light curve, the radio flux is increased by nearly an order of magnitude by the interaction. This is short of the ≥ 100 factor that is needed, so that mechanisms particular to the interaction are needed to produce the radio emission. The fraction f must increase by a factor of ~ 5 .

One mechanism for flux enhancement is particle acceleration by the reflected shock wave. If there is efficient electron heating in the original collisionless shock wave, the preshock electrons are semi-relativistic, so that the efficiency of electron acceleration to relativistic energies may be especially high. The fact that the observed spectrum flattened from an initially very steep spectrum¹ suggests that particle acceleration was taking place. The standard theory of shock acceleration¹⁴ predicts the spectral index of the particles once an equilibrium spectrum is attained. For the expected 1.86 density jump in the weak shock, the expected radio spectral index would be -1.75 . The spectral index was observed¹ to be steeper than -1.6 on day 1,243, but it rapidly became flatter. Although the standard shock acceleration theory can explain the initial spectrum, additional processes are needed for the later, flatter spectrum.

Another possible factor is the action of the Richtmyer-Meshkov instability¹⁵ when the supernova shock reaches the density discontinuity. This instability is equivalent to the Rayleigh-Taylor instability for an impulsively accelerated density discontinuity, and it is expected to lead to turbulent mixing in the vicinity of the interface¹⁶. A related process is that if the shock front and density interface are not parallel, vorticity is generated near the density interface. It is plausible that the magnetic field is amplified and particle acceleration may also occur.

The required efficiency of these processes can be estimated from standard minimum-energy arguments¹⁷. I take the outer shock radius to be 3×10^{17} cm in November 1990. If the interaction is with a free wind, the inner shock radius is $R_i = 2.4 \times 10^{17}$ cm, giving an estimate of the emitting volume. For Arnett's supernova density distribution⁷, the internal energy in the shocked layer is 3.1×10^{47} erg; this energy is $\propto R_i^{-4.6}$. For an outer radius of 3×10^{17} cm, a filling factor of 0.50, and a radio luminosity of 3×10^{32} erg s⁻¹ (ref. 1), the minimum energy in magnetic fields and relativistic electrons is 2.6×10^{45} erg; the minimum energy is increased to 3.7×10^{46} erg if there is 100 times the energy in relativistic protons that there is in relativistic electrons. The overall energy requirements are thus reasonable, although very high efficiencies of particle and magnetic field production may be needed if the radio-emitting volume is a fraction of the total shocked volume. Efficient proton acceleration leads to γ -ray emission by pion decays, but the maximum predicted flux is below that detectable by current techniques.

The timescale for the reflected shock front to traverse the shell in one place is ~ 6 months. The wind termination shock is unlikely to be exactly circular, but is probably somewhat elongated along the symmetry axis of the line-emitting ring, thus spreading out the emission timescale. The light travel time across the entire shell is 0.6 yr, which further spreads the emission in time. An estimate of the rise time for the emission is one year. Once the reverse shock front has traversed the interaction region,

the flow is expected to evolve toward a self-similar state for expansion in a roughly uniform medium, and the mechanisms that give rise to the enhanced emission no longer operate. The efficiencies for the production of radio synchrotron emission may return to values comparable to those present for the wind interaction, although continued interaction with the constant-density shocked blue supergiant wind should eventually give a slowly rising flux. The initial interaction with the dense ring is not expected to give a strong radio event because of the ring's small covering factor³.

The shock interactions can be expected to give X-ray emission. Extrapolation of the model for interaction with the blue supergiant wind² to November 1990 yields a luminosity of $\sim 1.5 \times 10^{34}$ erg s⁻¹ and a temperature of ~ 6 keV. The emission is primarily from the reverse shock front, which is first affected by the interaction with the termination shock by the reflected shock front. The resulting initial increase in X-ray flux is by a factor < 2 , but a slow increase follows because of the deceleration of the reverse shock front. The most sensitive X-ray observations are those made in December 1990 by the Broad-Band X-ray Telescope, which set an upper limit of 1.4×10^{35} erg s⁻¹ in the 2–10-keV band¹⁸, consistent with the estimates given here. In the present model, the radio 'flare' is not accompanied by one in X-rays, or in intermediate wavelength regions. $\square$

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Detection of helium-3 in a planetary nebula

R. T. Rood*, T. M. Bania† & T. L. Wilson‡

* Department of Astronomy, University of Virginia, Box 3818, Charlottesville, Virginia 22903, USA

† Department of Astronomy, Boston University, 725 Commonwealth Avenue, Boston, Massachusetts 02215, USA

‡ Max-Planck-Institut für Radioastronomie, Auf dem Hügel 69, D-5300 Bonn 1, Germany

HELIUM-3 is created by cosmological nucleosynthesis with an abundance $^3\text{He}/\text{H} \approx 2 \times 10^{-5}$ (ref. 1), but then augmented by stellar nucleosynthesis. Stars comparable in mass to the Sun should contribute a large fraction of the present ^3He abundance in interstellar material²; winds from these stars during their main-sequence lifetime, as well as planetary nebulae created by more rapid mass loss later in the stars' lives, are expected to have $^3\text{He}/\text{H}$ about 100 times the cosmic value. These stars are also thought to be the principal source of new material to the interstellar medium³, and measurement of the present ^3He abundance should therefore be an important diagnostic of chemical evolution in the Galaxy,

techniques for dealing with and estimating the size of these errors in Green Bank data, the errors are instrument-specific. Our limited experience with the 100-m telescope leaves several concerns unresolved because (1) the larger telescope leads to smaller-scale baseline structure which is comparable in size to the line widths, (2) 'standard' observing techniques might lead to some baseline structure which we do not yet understand and (3) some of the results are not what would be naively expected. In particular, the standard wisdom^{8,9} is that recombination lines from high- n states in planetary nebulae should be extremely broad and weak. Yet we find the H171 η line with intensity similar to the H114 β line. Further, the line centres of the H91 α and H171 η lines are $\sim 10 \text{ km s}^{-1}$ different from that of $^3\text{He}^+$. (The larger line width for the $^3\text{He}^+$ is not a major concern. The $^3\text{He}^+$ intensity is proportional to the density, and the recombination lines to the density squared. Hence, one would not necessarily expect the same line shapes, discounting such factors as pressure broadening.) Despite these concerns, we note that in observing literally thousands of weak lines we have not seen a line as 'good' as the NGC3242 $^3\text{He}^+$ line 'go away'. There is little question of the reality of the line, but single-epoch observations can give very inaccurate line parameters (compare values for W3 in refs 6 and 7), and the real line strength could be considerably smaller than the quoted value.

Even with well-determined line parameters, it is nontrivial to arrive at an abundance in terms of the $^3\text{He}/\text{H}$ ratio⁷. The ratio depends on the radio continuum, the source size and distance, and a model of source structure. Our angular resolution is not sufficient to determine the size, so we have adopted a size of $25''$ on the basis of H α profiles^{10,11}. Distance estimates¹²⁻¹⁷ range from 0.5 kpc to 2.4 kpc. We have adopted 1 kpc. We assume an electron temperature of 15,000 K. We assume NGC3242 to be a homogeneous sphere, as we do not yet have the required high-angular-resolution data. Experience in modelling other sources has shown that this might lead to errors of a factor of 2.

With our observed line parameters, the derived abundance is

$$^3\text{He}/\text{H} \approx 3 \times 10^{-3} \left(\frac{\theta}{25''} \right)^{-1.5} \left(\frac{d}{1 \text{ kpc}} \right)^{-0.5}$$

The mass of ionized hydrogen is

$$M_{\text{H}^+} \approx 0.5 \left(\frac{\theta}{25''} \right)^{1.5} \left(\frac{d}{1 \text{ kpc}} \right)^{2.5} M_{\odot}$$

The r.m.s. electron density is

$$n_e \approx 1.8 \times 10^3 \left(\frac{\theta}{25''} \right)^{-1.5} \left(\frac{d}{1 \text{ kpc}} \right)^{-0.5} \text{ cm}^{-3}$$

The planetary nebula from a $1 M_{\odot}$ star of solar abundance should have $^3\text{He}/\text{H} \approx 7 \times 10^{-4}$, and the $^3\text{He}/\text{H}$ ratio is expected to increase with main-sequence lifetime of the progenitor¹. Our candidate objects were selected to be far from the galactic plane ($b^{\text{II}} = 32^\circ$ for NGC3242) to increase the likelihood of a low-mass progenitor. For such stars, $^3\text{He}/\text{H}$ could be $\geq 10^{-3}$. These expected abundances are a factor of a few less than the value resulting from our adopted parameters. Given the uncertainties, we consider our result to be consistent with theoretical expectation.

We now have direct evidence that the ^3He produced in the outer parts of stars with mass $\sim 1 M_{\odot}$ during main-sequence hydrogen burning, and mixed to the surface during the red-giant phase, survives until the ejection of a planetary nebula. A survey of ^3He in planetary nebulae will place constraints on the lower mass limit to hot-bottom-envelope¹⁸ burning found in intermediate-mass stars which could destroy ^3He during the asymptotic-giant-branch phase. It will provide direct evidence of the rate and location of stellar ^3He input to the interstellar medium. If NGC3242 is typical, ^3He is being added to the interstellar medium at a significant rate, and models for chemical evolution of the Galaxy will have to satisfy an additional constraint.

Arriving at a primordial abundance may require large evolutionary corrections to H II region abundances. $\square$

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Isotope effect on superconductivity in Rb_3C_{60}

T. W. Ebbesen, J. S. Tsai, K. Tanigaki, J. Tabuchi, Y. Shimakawa, Y. Kubo, I. Hirose & J. Mizuki

Fundamental Research Laboratories, NEC Corporation, Tsukuba 305, Japan

THE surprisingly high transition temperatures (T_c) for superconductivity in alkali-metal-doped C_{60} has spurred wide interest in understanding its mechanism¹⁻⁶. Recently the increase in T_c with lattice constant was demonstrated for these materials⁶, and was interpreted as resulting from the corresponding increase in the density of states at the Fermi level. According to the standard (BCS) theory of superconductivity, the other important factor controlling T_c is the phonon that mediates electron pairing. To test whether this factor plays a part for the C_{60} superconductors, we prepared C_{60} containing various amounts of ^{13}C , which we then doped with rubidium to give Rb_3C_{60} . Measurements of diamagnetic shielding and Meissner effect show that T_c decreases as the ^{13}C content increases, as expected within the context of BCS-like phonon-mediated pairing; but the dependence on the mass is stronger than for most electron-phonon superconductors where $T_c m^{-\alpha}$ with $\alpha \leq 0.5$. Instead, the exponent α has the remarkably large value of 1.4 ± 0.5 . Regardless of the interpretation of this value, it is clear that phonons have an important role in the origin of superconductivity in doped C_{60} .

We prepared ^{13}C -labelled C_{60} by slightly modifying the Krätchmer-Huffman method^{7,8}. Holes were drilled in the graphite rods consumed in the arc plasma experiments, and filled with amorphous ^{13}C . The average $^{13}\text{C}/^{12}\text{C}$ ratio in the C_{60} is determined by the isotope weight ratio in the feed, as C_{60} is grown in the vapour phase from thoroughly mixed carbon atoms⁸. The C_{60} masses follow a random statistical (Poisson) distribution (Fig. 1) and the spread was checked by mass spectrometry and infrared absorption. The labelled fullerenes were extracted from the soot with benzene, and C_{60} was purified by column chromatography. The samples were then doped with rubidium for superconductivity measurements. The desired quantity of rubidium was obtained by cutting the appropriate length of rubidium-filled capillary tubes in a helium-filled glove box. The reagents were inserted in quartz tubes which were then sealed and heated for three days at 400°C . The ratio of rubidium to C_{60} in the feed was always 3 to 1. All samples in a series were prepared in the same way, using the same conditions at the same time. The temperature dependence of d.c. magnetization was measured with a SQUID (superconducting quantum

interference device) magnetometer (Quantum Design MPMS). The infrared spectra were recorded on a Perkin-Elmer FTIR 1725 X.

The effect of ^{13}C on the vibrational features of C_{60} was first checked by infrared absorption spectroscopy. The spectrum of C_{60} with various fractions of ^{13}C is always characterized by four peaks. Because of the random statistical distribution of isotope content (and hence C_{60} masses), the absorption peaks become broader as the isotope mixture approaches 50% (ref. 8). The infrared spectrum and the mass distribution for a sample with 33% ^{13}C content are shown in Fig. 1. Furthermore, the absorption frequencies shift to shorter wavenumbers with increasing ^{13}C content. In the inset to Fig. 1, the peak absorption frequency is plotted against $m^{-1/2}$ where m is the average mass of C_{60} for a given ^{13}C content. The slope is linear as expected.

The d.c. magnetization measurements, both field-cooled and zero-field-cooled, were done for similarly prepared rubidium-doped samples. Onset temperatures of superconductivity (T_c) were always the same for shielding signals and Meissner signals for a given sample (Fig. 2, inset). Figure 2 shows the temperature dependence of the Meissner signal near T_c for three different ^{13}C contents. Clearly T_c depends strongly on the ^{13}C content, decreasing by as much as 1 K when the average mass of C_{60} increases from 720 to 740 (33% ^{13}C). The gradient dM/dT , where M is magnetization, near T_c was nearly linear, as expected from the Ginzburg-Landau formalism⁹. Thus T_c was defined by the linearly extrapolated intercept temperatures, using the slope dM/dT . We also used another definition of T_c , the onset temperature where the d.c. magnetization starts to deviate from the background, to calculate the isotope effect and check for consistency. Although the absolute magnetization varied a little from run to run (shielding fractions were typically $\sim 10\%$), the advantage of this material is that for the standard reference samples, T_c is always reproducible. Here we used three reference samples with natural ^{13}C content, prepared at different times. The reproducibility of T_c was established within ± 0.1 K (Fig. 3). This error margin is an order of magnitude smaller than the observed change induced by the increased C_{60} mass.

To interpret our result, we can consider the standard electron-phonon BCS model which predicts that T_c should be very sensitive to the phonon frequencies, as they are expected to

mediate the electron pairing. According to BCS theory, T_c is given by:

$$T_c = \omega e^{-1/gN(E_F)} \quad (1)$$

where ω is the phonon cut-off frequency of the phonons mediating the electron coupling, g is the electron-phonon coupling strength and $N(E_F)$ is the density of states per unit energy at the Fermi level. In the first instance, if we assume that $N(E_F)$ and g remain constant, we could expect that T_c is a function of the vibrational frequencies of C_{60} (that is, ω), and therefore a function of $m^{-\alpha}$, as for the infrared frequencies (Fig. 1). Figure 3 shows such a plot in which $\ln T_c$ has been plotted against $\ln M$ to determine α . When extrapolated values of T_c are used the slope gives $\alpha = 1.4 \pm 0.5$. The error margin (and the error bars in Fig. 3) reflect the half-width of the C_{60} mass distribution as shown in Fig. 1. When 'onset' values of T_c are used, α is $\sim 6\%$ larger, indicating that the interpretation of the data is consistent. A similar plot can be made by taking into account the mass of the rubidium, but the slope becomes even larger. On the experimental side, it is important to note that the presence of C_{60} with mass centred on pure ^{12}C in addition to the ^{13}C -enriched C_{60} (that is a mass spectrum with two peaks, a common problem when preparing enriched C_{60}) significantly lowers α , because the small ^{12}C -centred superconducting fraction gives rise to a small but higher onset in the magnetization-temperature ($M-T$) curve. The resulting $M-T$ curve mimics that of a single-phase material with T_c in the range $T_c(^{13}\text{C}/^{12}\text{C}) < T_c < T_c(^{12}\text{C})$. For instance, we found that when 10–20% of the total mass was due to C_{60} centred on ^{12}C (double peak in the mass spectra), α was typically ~ 0.4 simply because the apparent onset is then shifted to higher temperatures.

From the large value of α , T_c is not a simple function of mass affecting either the intramolecular or intermolecular phonons as seen from the vibrational frequencies of C_{60} (Fig. 1). So in the context of electron-phonon BCS theory, both ω and λ ($\lambda = gN(E_F)$) might be strongly influenced by the phonon

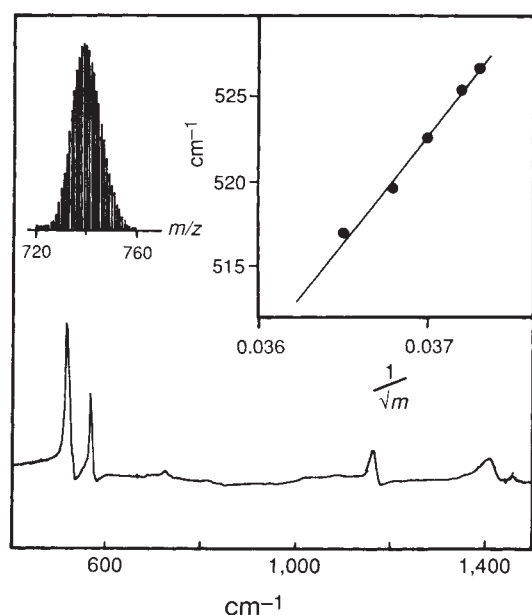


FIG. 1 Infrared spectrum of C_{60} containing 33% ^{13}C , and its mass distribution. Inset: dependence of the infrared peak position on the average mass of C_{60} .

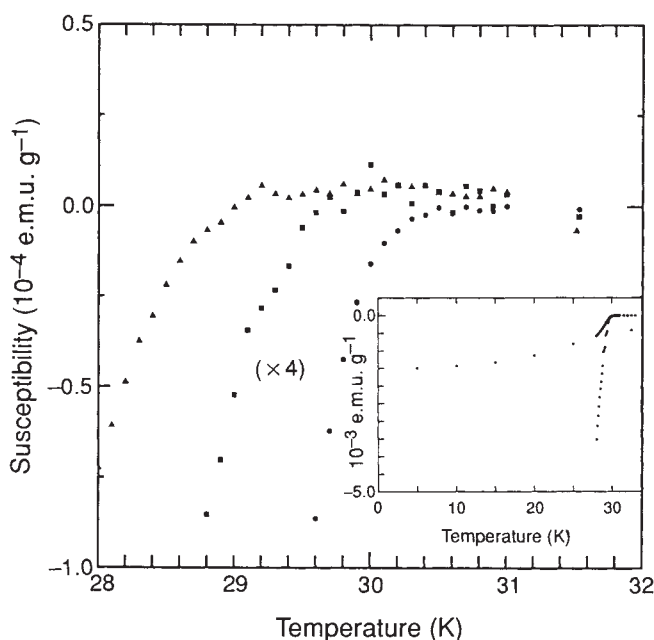


FIG. 2 Meissner (field-cooling) curves as a function of temperature showing in detail the T_c onset area for three different ^{13}C content, (●): 1.1%; (■): 31%; (▲): 33%. The curve for 31% has been multiplied by 4 to separate it more clearly from the 33% curve. Inset: zero-field-cooled and field-cooled magnetization curves for C_{60} with natural isotopic composition (1.1% ^{13}C).

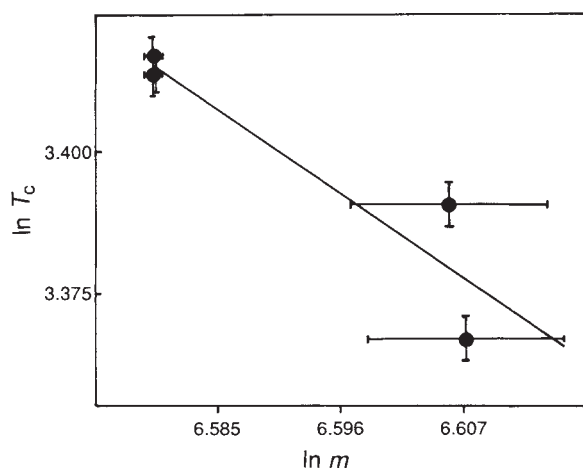


FIG. 3 Relationship between $\ln T_c$ and $\ln m$ (average C_{60} mass). The large horizontal error bars are due to the statistical spread of the C_{60} masses at a given average ^{13}C content.

frequencies of the system. For materials where the strength of the electron-phonon coupling λ is <1.25 , α should always be smaller than 0.5, according to the '2 square model' as well as the McMillan approximation where a Coulomb repulsion is taken into account in addition to the phonon attraction^{10,11}. But tunnelling spectroscopy of Rb_3C_{60} reveals a well defined superconducting energy gap of energy $2\Delta = 5.3 kT_c$ (ref. 12). It is conceivable that the 'real' energy gap is even larger, considering the enhanced surface proximity effect for materials of short coherence length¹³ such as the alkali-doped C_{60} (refs 14, 15). In any case, the reduced energy gap of $5.3 kT_c$ is even larger than that of strongly coupled superconductors such as lead and mercury, whose reduced energy gaps are 4.38 and 4.6 respectively. The coupling, λ , for lead is 1.12 and that for mercury is 1.0. Thus for Rb_3C_{60} , λ might very well be larger than 1.25, making the analysis given above for $\alpha \leq 0.5$ not quite valid. For higher values of λ ($1.25 < \lambda < 15$), there may be a numerical solution of α larger than 0.5 (ref. 11). Moreover, the conventional treatment of the Eliashberg equation which gives rise to the analysis of T_c and α in several limits of λ (ref. 11) may not be satisfactory in doped C_{60} materials¹⁶.

The large α might be partly explained by changes in lattice constant, as the presence of ^{13}C will slightly shrink the intramolecular covalent carbon-carbon bonds in C_{60} . But as the crystal is ionic rather than covalent, changes in the intramolecular carbon bonds probably result in little change in the lattice dimensions. We tried to measure any changes in lattice constant but could detect none within our experimental error. The resolution of our equipment (0.01 Å) and the broad distribution of masses in the sample, however, made it difficult to measure the lattice constant accurately; further measurements would be welcome on this point.

The large value of α obtained, the non-exponential dependence of T_c on the density of states $N(E_F)$ (ref. 16) and the unusually strong coupling¹² may indicate that the simple BCS picture is not applicable for this new series of materials. Nevertheless, taken together with the results in ref. 6, it is now clear that both the lattice dimensions (and thereby the density of states at the Fermi level) and the phonons are key parameters controlling the superconductive properties of alkali-doped C_{60} . *Note added in proof:* Since submission of this paper, two sets of results have been brought to our attention which are consistent with our observations. A. P. Ramirez *et al.* report in a preprint an α of 0.32 for a sample containing 15% C_{60} centred on ^{12}C (double peak mass spectrum) and Zakhidov, Yakushi, Achiba and their co-workers have found a value of α in the range 1.4–1.8 for a sample which has a single mass distribution but at a much higher degree of enrichment than ours. □

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The structure of the C_{70} molecule

D. R. McKenzie*, C. A. Davis*, D. J. H. Cockayne†, D. A. Müller*‡ & A. M. Vassallo‡

* Department of Applied Physics, University of Sydney, New South Wales 2006, Australia

† Electron Microscope Unit, University of Sydney, New South Wales 2006, Australia

‡ CSIRO Division of Coal and Energy Technology, PO Box 136, New South Wales 2113, Australia

ALTHOUGH there have been experimental studies of the structure of the C_{60} molecule in the gas phase¹ and the solid state at low temperature², so far only indirect experimental details of the structure of the C_{70} molecule have been available³. The ellipsoidal structure for C_{70} , the subject of recent theoretical work^{4,5}, should provide a useful test of current understanding of carbon-carbon bonding, in that a single molecule contains several distinct types of bond in various relationships to pentagonal and hexagonal rings. Conventional crystallographic techniques are not suitable for studying C_{70} at room temperature because of poor crystallinity and orientational disorder in solid samples. Here we present the results of a study in which the molecular structure of C_{70} was deduced from electron diffraction using a simulated-annealing method. The 'rugby ball' structure is confirmed, with a slight pinching of the waist, and the bond lengths are found to follow a simple pattern determined by their relationship to the five- and six-membered rings.

Electron diffraction techniques⁶ enable the scattered intensity from selected areas of small samples to be measured with good counting statistics to large values of $s = 2 \sin \theta / \lambda$ (here $s \leq 4.7 \text{ Å}^{-1}$). In the presence of rotational disorder, structural information can be obtained from the reduced density function $G(r) = 4\pi r(\rho(r) - \rho_0)$, where $\rho(r)$ is the density of atom centres at a distance r from an average atom, and ρ_0 is the average density.

Data were collected with a Philips EM430 electron microscope operating at 300 kV. An electron energy-loss spectrometer (GATAN 607 PEELS) was used to detect only elastically scattered electrons. A sample containing 10% C_{70} and 90% C_{60} was prepared from a graphite arc source operating in helium. The C_{70} fraction was separated by liquid-phase chromatography of a toluene solution. The purified C_{70} contained more than 90% C_{70} with the remainder being mostly graphite. A thin film was then deposited by vacuum evaporation onto a cleaved NaCl crystal surface, removed by flotation and mounted on a copper grid. The deposited film is estimated to contain more than 97% C_{70} , as the evaporation acts as a further purification step, leaving the graphite contamination behind.

The molecular structure of C_{70} can be obtained from the $G(r)$ of Fig. 1 using a procedure⁷ in which the atoms in a simulated molecule are assumed to interact through a potential equal to $-G(r)$, which approximates the 'potential of mean force' of two

atoms in an ensemble⁸. The molecular structure with lowest total energy is found by relaxing the computer-generated molecule using a simulated annealing algorithm. This procedure uses the information available in $G(r)$ primarily through the positions of its maxima and leads to a molecular structure in a natural way, with the only constraint being the experimental data. The technique has a special advantage for electron diffraction in that multiple scattering (not severe for the 300-kV beam and the 200-nm film thicknesses used here) affects the detailed shape of $G(r)$ but does not change the position of maxima⁹. The technique of fitting experimental data (either the diffraction intensity¹⁰ or the $G(r)$ (ref. 1)) by varying structural parameters is more sensitive to the effects of multiple scattering. The simulated annealing algorithm has been used previously to fit the neutron diffraction intensity of molten salts¹⁰.

Seventy atoms were arranged in a simulated structure having the form of a rough C_{70} molecule with the correct topology. The three coordinates of each of the seventy atoms were allowed to vary without any symmetry constraint. The atoms were given many small random displacements. If a displacement reduced the total energy then it was accepted; if not, it was occasionally accepted, with a probability $p = \exp(-\Delta U/kT)$, where ΔU is the energy increase and T is temperature. A series of values of kT was chosen to allow the structure to relax into a minimum energy state at low kT . The final structure was not sensitive to the choice of the annealing 'temperatures' T or to the bond lengths of the starting molecule.

We explored the accuracy of the method by using it with $G(r)$ calculated from known test structures. It is readily shown that the correct structure gives a local minimum in the energy calculated from the potential defined above, provided that $G(r)$ is not broadened by thermal, instrumental or termination effects. We confirmed this numerically, using several test structures. Although there is no guarantee that the structure found is unique, in practice the procedure always converged to the correct structure. In any practical application, $G(r)$ will be broadened. Such broadening introduced systematic shifts in the bond lengths from those of the test structure used to generate the $G(r)$. These shifts, at the broadening corresponding to our experimental data, were used to determine the uncertainty in the bond lengths of the final molecule.

The final relaxed molecule shown in Fig. 2 is derived from the experimentally measured $G(r)$ and had only small deviations from the expected D_{5h} symmetry even though symmetry was not imposed on the atom positions. This gives considerable confidence in the method. There are eight different types of

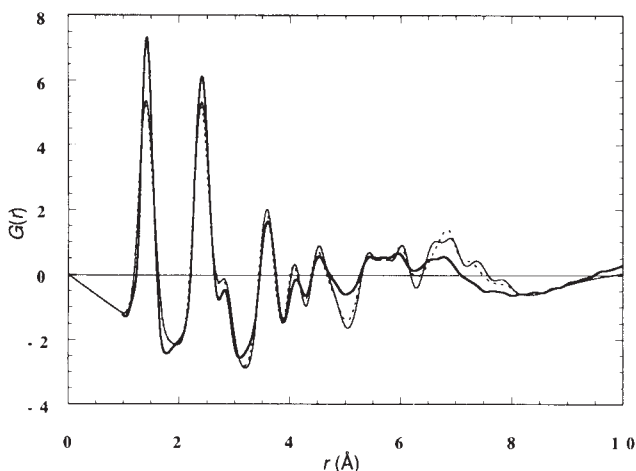


FIG. 1 Experimentally measured $G(r)$ for a thin film of C_{70} , obtained at 300 K (heavy line) compared with $G(r)$ calculated from the structure obtained by simulated annealing (medium line) and with $G(r)$ calculated from the theoretical molecule of ref. 5 (broken line).

TABLE 1 Average bond lengths

Bond type	Number of bonds	Category	Bond length (Å)
1	5	hexagon-hexagon (not within pentagon)	$1.41^{+0.03}_{-0.01}$
2	20	hexagon-pentagon	1.39 ± 0.01
3	10	within pentagon	$1.47^{+0.01}_{-0.03}$
4	20	within pentagon	1.46 ± 0.01
5	10	pentagon-pentagon	1.37 ± 0.01
6	20	within pentagon	1.47 ± 0.01
7	10	pentagon-pentagon	1.37 ± 0.01
8	10	within pentagon	1.464 ± 0.009

Average bond lengths and their uncertainties for each of the types of bond shown in Fig. 2 which are not related by D_{5h} symmetry. The four categories into which the bonds are grouped are also shown.

bonds labelled in Fig. 2 which are not related by symmetry. The numbering of the bonds gives a larger number the further the distance from the equator.

The distribution in bond lengths within a symmetry-related group gives a lower bound on the uncertainty in the length for that bond type. The other contribution to the uncertainty is the effect of broadening of $G(r)$ discussed above. We show in Table 1 the mean length of each bond type together with the estimated uncertainty. The eight bond lengths fall into four size ranges. The bonds defining a pentagon (types 3, 4, 6, 8) are long, with a mean value of 1.46 Å , which is equal to our previously determined value⁷ for the equivalent bond in C_{60} . The bonds joining the apices of two pentagons (types 5 and 7) are short, with a mean value of 1.37 Å , equal to our value⁷ for the equivalent bond in C_{60} .

Johnson³ has discussed the effects of the pentagons in C_{70} on bond lengths. An increase in the p character of the bonding within a pentagon results from the smaller pentagonal bond angle, and increases the length of these bonds. This effect in turn leads to an increase in the s character of bonds leading from a pentagon and hence to a decrease in their length. This prediction is obeyed by our results, and furthermore, the effects have the same magnitude in C_{70} as in C_{60} for bonds with the same environment. The remaining bonds in C_{70} have no equivalent in C_{60} . The five bonds around the equator (type 1) are not distorted by the proximity of pentagons. These bonds have a length of $1.41^{+0.03}_{-0.01} \text{ Å}$ which is the same as the graphite value of 1.42 Å . The asymmetrical uncertainty results from the

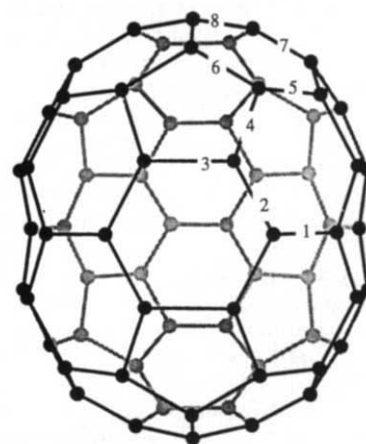


FIG. 2 The C_{70} structure calculated from the experimental data. The eight types of bond which are not symmetrically equivalent are enumerated in order from the middle of the molecule upwards. The pinching of the waist is a real effect, although visually exaggerated by the curvature of the hexagons spanning the equator.

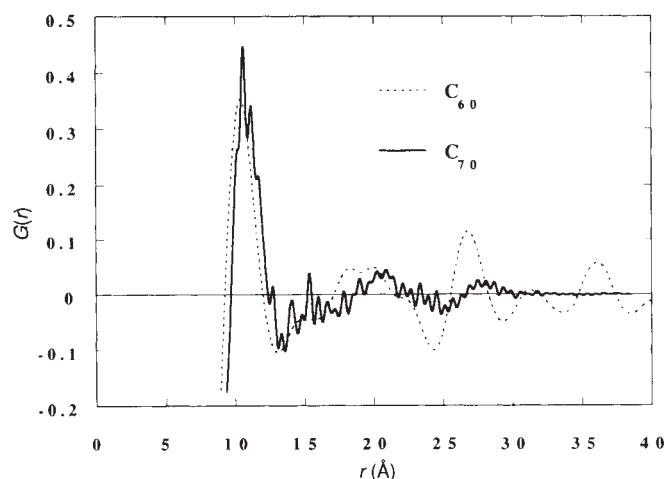


FIG. 3 $G(r)$ for C_{70} compared with $G(r)$ measured for C_{60} at large r . The two molecules show some similarities in their packing, although C_{70} is larger and shows far less long-range order.

effects of experimental broadening of $G(r)$. The 20 near-equator bonds (type 2) join the apex of a pentagon to the apex of a hexagon and have a length of 1.39 ± 0.01 Å. This is intermediate in length between the short bonds joining two pentagons and the graphite-like bonds joining two hexagons. Thus the variations in our bond lengths fit a simple pattern, obeying the rule discussed by Johnson³ well.

The predictions of the *ab initio* Hartree-Fock calculations of Scuseria⁴ are generally consistent with our results with two important exceptions. He finds bond lengths for type 1 bonds of 1.475 Å, and for type 3 bonds of 1.415 Å. This large value for the equator bonds (type 1) is not consistent with our value nor with a graphite-like central region of the molecule. The small value calculated for type 3 bonds is equally puzzling, because the pentagon containing them is surrounded by five hexagons, as is the case for all other pentagons in the structure, and it is difficult to see why this bond type should have a different length. A second theoretical calculation⁵ gave similar results to those of Scuseria, and atomic coordinates were quoted. We found that, even with broadening effects included, the length of equator bond in the theoretical molecule of Baker *et al.*⁵ was inconsistent with our observations. In addition, the observed intensity and the observed $G(r)$ were better fitted by our proposed structure than by the theoretical prediction (see Fig. 1).

The overall height of the relaxed molecule, defined as the distance between the planes of the two pentagonal end faces, is 7.80 ± 0.01 Å. The diameter of the equator, defined as the diameter of a circle passing through the centre of equatorial atoms, is 6.94 ± 0.05 Å. A circle drawn through all atoms one layer away from the equator, connected by type 3 bonds (see Fig. 2), has a diameter of 6.99 ± 0.05 Å, indicating that the molecule is slightly 'pinched' around the middle. This tendency for the molecule to form a dumbbell shape may reflect the stability of a spherical shape for the C_{60} -like end caps.

The function $G(r)$ also contains useful information on the packing of C_{70} molecules in the solid. In Fig. 3 we show the $G(r)$ out to 40 Å together with our earlier result⁷ for C_{60} . A reduced long-range order in C_{70} compared with C_{60} is readily apparent as a loss of signal in $G(r)$ beyond 32 Å. This is probably because the non-spherical shape of the C_{70} molecule gives rise to a larger density of defects in the molecular packing. The general shape of $G(r)$ for $10 < r < 30$ Å is somewhat similar to the $G(r)$ for C_{60} with an allowance for the larger molecular dimensions of C_{70} . The packing of the molecules could be described as defective close-packed layers with a high degree of stacking disorder. □

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Photochromism induced in an electrolytically pretreated MoO_3 thin film by visible light

J. N. Yao, K. Hashimoto & A. Fujishima

Department of Synthetic Chemistry, Faculty of Engineering, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

PHOTOCHROMIC materials, whose optical absorption properties change in response to light, are important for a number of technological applications. A stable, reusable photochromic thin-film could be used, for example, in optical displays and high-density memories. Thin films of some transition-metal oxides can be coloured blue by band-gap excitation using ultraviolet light^{1–6}. For use with common semiconductor laser sources, however, photochromic materials are required that respond to visible light. Here we report on the preparation of visible-light-sensitive, reversibly photochromic films of MoO_3 . Pretreated, vacuum-evaporated MoO_3 thin films were slightly blued by cathodic polarization in a non-aqueous electrolyte; subsequent irradiation with visible light in air produced a strong colour enhancement. The photochromism could be erased by anodic polarization, and the coloration–decoloration process was repeatable over at least five cycles. The behaviour of these films contrasts with those blued by band-gap irradiation, which were not responsive to visible light.

The MoO_3 amorphous film was prepared by vacuum evaporation of high-purity MoO_3 powders (99.9%) onto a 1-mm-thick NESA glass substrate (5×2.5 cm), with a typical film thickness of $\sim 1,000$ nm being obtained. Photochromic experiments were done in air, and a 500-W high-pressure mercury lamp was used as a light source. For band-gap excitation, the light fell directly on the sample, whereas for the visible-light experiments a sharp cut-off filter was used to obtain light of wavelength ≥ 500 nm. The size of the illuminated area was varied from ~ 1 mm² to 1.8 cm², but this did not affect the characteristics of the photochromic response. In the electrochromic experiments, the sample was dipped into 0.1M LiClO_4 /propylene carbonate solution. Platinum and Ag/AgCl electrodes were used as counter and reference electrodes, respectively. The surface area of the MoO_3 film in contact with the electrolyte was 1.8 cm². Because we did not add any redox couple into the electrolyte, the reactions occurring at the counter-electrode were probably the reduction and oxidation of water present as an impurity in the electrolyte.

Because freshly prepared MoO_3 is colourless and transparent, it does not show photochromism on irradiation by visible light. After being slightly blued by electrochromism, however, it becomes sensitive to visible light. Figure 1a shows the changes in the absorption spectrum of the MoO_3 thin film, with spectra for both the prepared amorphous film and the electrolytically pretreated film being shown. This result was obtained as follows. First, the absorption spectrum of the prepared MoO_3 colourless film was recorded (curve A). Next, the film was polarized at -0.1 V versus Ag/AgCl for 3 min in the electrolyte. The film was slightly coloured, as indicated by curve B. The change in absorbance from curve A to curve B was ~ 0.05 at 800 nm. This electrolytically pretreated film was then irradiated with visible

light for 10 min in air. The irradiated part was blued deeply (curve C): the change in absorbance was ~ 0.3 at 800 nm. Similar colour enhancement was also observed on near-infrared irradiation ($\lambda \geq 700$ nm). This result indicates that even when excited by a broad absorption band (500–1,000 nm), the photochromic reaction is induced in this pretreated MoO_3 film. The possibility that this colour enhancement was caused by a thermal process could be excluded because the colour enhancement induced by visible light was affected neither by putting a thermal cut-off filter between the cell and the light source nor by doing the irradiation at 80°C.

Figure 1b shows, in contrast, that a MoO_3 film blued by band-gap irradiation pretreatment was not sensitive to visible light irradiation in air, even though the absorption spectrum of a film blued in this way (curve E) is very similar in shape to that of a film blued by the electrochromic reaction.

Photochromism induced in MoO_3 thin films by visible light has not, to the best of our knowledge, been reported previously. After coloration by visible light irradiation, the MoO_3 thin film was exposed in air for a prolonged period. No absorbance change was detected from films continuously exposed in air for seven months after the initial coloration, indicating that the coloured film was very stable in air.

The MoO_3 film coloured by visible light irradiation also showed good reversibility in its coloration–decoloration process. This was confirmed by the following experiments, done at constant current. First, the film was slightly blued by electrochromism with a current density of $-5.5 \mu\text{A cm}^{-2}$ for 17 min, then irradiated by visible light for 15 min. For decoloration, this deeply coloured film was polarized anodically with a current density of $+5.5 \mu\text{A cm}^{-2}$ for 34 min. The resulting bleached sample was still slightly coloured. Therefore it was deeply coloured again by visible light irradiation and decoloured repeatedly by the same procedure. The results are summarized in Fig. 2. If the film was completely decoloured, it was no longer sensitive to visible light. But if the colourless film was slightly blued again, it became sensitive to visible light.

The conventional photochromic processes is explained as follows³. When a thin film of MoO_3 is irradiated by ultraviolet light, electrons and holes are formed, thereby allowing the photogenerated holes to react with surface-adsorbed species, causing the film to be negatively charged. The positive ions on the surface, protons in the present case, are then injected into the film by Coulomb attraction, forming the hydrogen molybdenum bronze. The observed visible-light-induced photochromic reaction might, however, originate from a different reaction mechanism. The broad absorption band in the visible

region is due to the charge-transfer transition from Mo^{5+} to the oxide ligands, producing Mo^{6+} (refs 7, 8). Therefore, it is apparent that the excitation of this broad band does not produce electron–hole pairs in the film. We suggest that the MoO_3 film changes to an intermediate metastable state during the electrolytic reaction and is then converted by visible light into stable molybdenum bronze.

At present the origin of the intermediate metastable state is not known, although the key to the problem is probably that the visible-light-sensitive photochromism is observed only with films slightly blued by the electrochromic reaction and not by the conventional band-gap photochromic reaction. One possible explanation is based on the difference between the sizes of the positive ions in the bronze. The injected positive ions for the electrochromic and photochromic reactions are Li^+ and H^+ , respectively. Because Li^+ is much larger than H^+ , the structural changes are much larger in films coloured by the electrochromic reaction than in those coloured by the photochromic reaction. Therefore when Li^+ is injected into the film, its structural distortion may result in a unique intermediate metastable state which can be converted to the stable bronze structure by visible irradiation. Another explanation is based on the energy difference between optical excitation and electrolytic polarization. The optical band-gap excitation energy is enough to produce stable

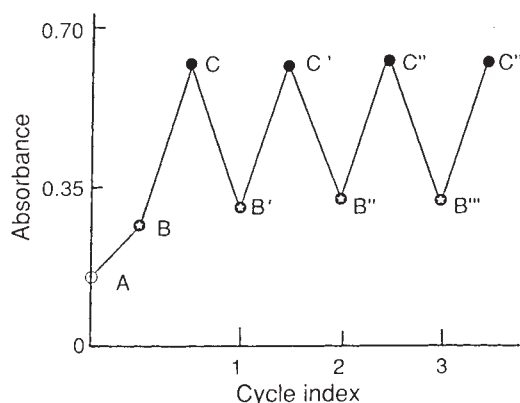


FIG. 2 The absorbance change (Δ_{abs}) of MoO_3 thin film at 800 nm during coloration and decoloration. The electrochemical polarization was done at constant current. A, Taken after a freshly prepared MoO_3 thin film was repeatedly polarized four times cathodically ($-5.6 \mu\text{A cm}^{-2}$) and anodically ($+0.6 \mu\text{A cm}^{-2}$) for 17 min. B, Taken after film A was polarized cathodically ($-5.6 \mu\text{A cm}^{-2}$) for 17 min, or film C anodically ($+5.6 \mu\text{A cm}^{-2}$) for 35 min. C, Taken after B was irradiated with visible light for 15 min in air.

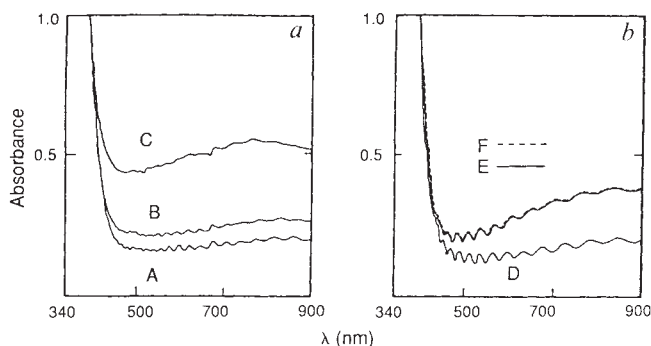


FIG. 1 Absorption spectra of a MoO_3 thin film. a, MoO_3 thin film as prepared by vacuum evaporation (curve A); spectrum taken after polarization at -0.1 V versus Ag/AgCl for 3 min in $0.1 \text{ M LiClO}_4/\text{propylene carbonate}$ solution (curve B); and taken after the film in curve B was irradiated with visible light ($\lambda \geq 500$ nm) for 10 min in air (curve C). b, MoO_3 thin film as prepared by vacuum evaporation (curve D); taken after D was irradiated with ultraviolet light for 8 min in air (curve E); and taken after E was irradiated with visible light ($\lambda \geq 500$ nm) for 10 min in air (curve F).

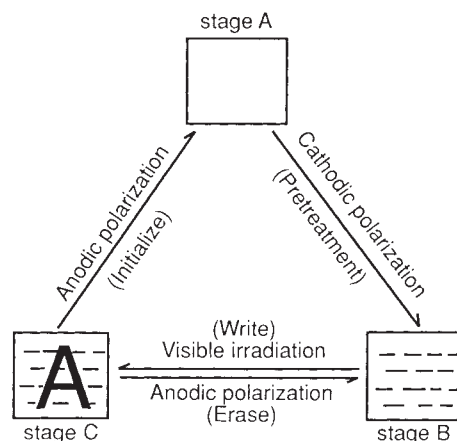


FIG. 3 Proposed application of the visible-light-enhanced photochromic reaction in display device.

molybdenum bronze directly, whereas that of the electrolytic reduction is not. Experiments to elucidate the mechanism are now being done.

Figure 3 shows a scheme using a combination of the 'electrochemical mode' and the 'photon mode' in a MoO_3 thin film to achieve a display device, for example. Starting with a colourless MoO_3 film (stage A), it is slightly blued by cathodic polarization (electrochemical mode) to generate a visible-light-sensitive material (stage B). Then the desired pattern is written on it by visible light irradiation (photon mode, stage C). This pattern is easily erased at will either to the pretreated stage (stage B) or to the initial stage (stage A) by controlling the time for anodic polarization, and the film can be used repeatedly. □

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Tropical stratospheric circulation deduced from satellite aerosol data

Charles R. Trepte & Matthew H. Hitchman

Department of Meteorology, University of Wisconsin–Madison,
1225 West Dayton Street, Madison, Wisconsin 53706, USA

THE dispersal of volcanic material from large tropical eruptions provides insight into the circulation of the lower stratosphere. Here we infer stratospheric motions from zonal mean cross-sections of satellite observations of the aerosol layer taken during 1979–81 and 1984–91. By examining the aerosol distribution following volcanic eruptions in the tropics, we find that poleward transport occurs readily at altitudes within a few kilometres above the tropopause, whereas in the altitude range of 21–28 km, aerosols tend to remain within 20° of the Equator. We further deduce that the aerosol distribution in this upper regime is controlled by the phase of the quasi-biennial oscillation. When the easterly shear is present, aerosols are lofted over the Equator, whereas when the westerly shear is present, descent relative to the mean stratospheric circulation occurs over the Equator. From the aerosol distributions, we suggest that the tropical stratosphere may be regarded as a temporary reservoir for trace constituents entering the stratosphere through the tropical tropopause.

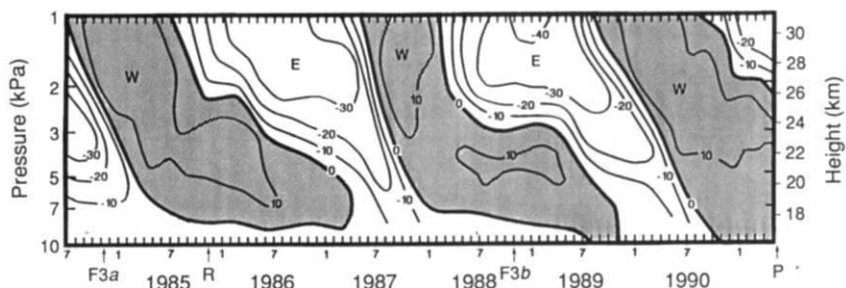
Stratospheric sulphate aerosols, primarily the byproduct of volcanic eruptions, have long been a focus of study¹. They can influence the climate by altering the distribution of radiative heating and may affect the ozone layer by liberating active

chlorine and repartitioning odd-nitrogen species². Here we use their distribution to deduce the circulation in the lower stratosphere. This circulation determines how air enters the stratosphere from the troposphere, and hence how trace constituents become distributed throughout the ozone layer.

From consideration of the water-vapour budget³, the column ozone distribution⁴, the radiative budget⁵, the dispersal of radioactive tracers^{6,7} from bomb tests in the 1950s and 60s, and the pattern of diminution of solar radiation at the surface⁸ after the eruption of Mount Agung in March 1963, the following pattern of mean stratospheric circulation has emerged and is now known as the Brewer–Dobson circulation. Air enters through the tropical tropopause. Some air travels poleward within a few kilometres of the tropopause and re-enters the troposphere in the extratropics, with the transport being stronger into the winter hemisphere. In the middle and upper stratosphere, air ascends further in the sunlit portion and descends over the winter pole. The circulation in the tropical lower stratosphere is dominated by a quasi-biennial oscillation (QBO) in zonal wind and temperature⁹. Figure 1 shows the variation of monthly mean zonal wind at Singapore for the period July 1984 to May 1991. The meridional circulation associated with the QBO, however, has only been estimated from theory and numerical models^{10–12}. We have examined satellite estimates of the ratio of aerosol to Rayleigh extinction near 1 μm (refs 13, 14) from the stratospheric aerosol and gas experiments (SAGE I and II)^{13,15} for the periods 1979–81 and 1984–91, respectively. Here we present sample distributions of sulphate aerosols and deduce the associated mean meridional circulation during two phases of the QBO (Fig. 3).

Detailed temperature measurements taken during November 1978 to May 1979 allow the QBO structure to be represented in terms of zonal mean potential vorticity (PV)¹⁶. PV can be regarded as a measure of the inertial and static stability of the air¹⁷. Where the gradient of PV is strong, the transport of constituents across that gradient will be inhibited¹⁸. The zonal mean distribution of PV is controlled by the absorption of gravity and planetary waves, the mean meridional circulation and radiative processes¹⁹. Figure 2 shows the distribution of normalized PV for early November 1978, defined as $-(f/|f|)(1/r)\bar{v}M$, where f is the Coriolis parameter, r is the distance to the Earth's rotation axis, and $\bar{v}M$ is the zonal mean angular momentum gradient evaluated at constant potential temperature¹⁶. At this time, descending westerlies near 35 km associated with the equatorial semiannual oscillation¹⁶ overlaid QBO easterlies in the layer 24–32 km, which in turn overlaid QBO westerlies below 24 km. Near 30 km, PV contours are spread out about the Equator and close together near 20° N and S. This pattern is compatible with easterly wave driving centred over the Equator with attendant divergent meridional flow into the subtropics. Near 22 km and 38 km, PV contours are pinched together over the Equator and the gradient is large near 10° N and S, a pattern which is compatible with westerly wave driving centred over the Equator and convergent meridional flow. In agreement with expectations from theory and models^{10–12} this PV pattern is compatible with ascent over the Equator in easterly shear and descent relative to the mean stratospheric circulation over the

FIG. 1 Time–altitude section of monthly mean zonal wind component at Singapore (1° N, 104° E) for July 1984 to May 1991, adapted from ref. 24, showing the quasi-biennial oscillation in the equatorial lower stratosphere, contour interval 10 m s^{-1} . Marked times R, P, F3a and F3b are the eruptions of Mounts Ruiz and Pinatubo and the aerosol cross-sections in Fig. 3a, b.



Equator in westerly shear. Near 20° N and S, vertical divergence occurs near the level of maximum equatorial easterlies. We use this model of the QBO meridional circulation together with the observed equatorial winds in Fig. 1 to interpret the aerosol distributions.

Two sections of aerosol extinction ratio during boreal autumn typify the descending westerly (Fig. 3a) and easterly (Fig. 3b) phases of the QBO. Values increase rapidly above the tropopause and peak over the Equator. Within a few kilometres above the tropopause, contours suggest poleward and downward transport, being more pronounced in the Southern Hemisphere following winter. Behaviour in this lower regime is in agreement with previous results³⁻⁸. Remarkably strong gradients occur near 20° N and S to altitudes exceeding 25 km. The double ear structure seen above 23 km in QBO westerly shear (Fig. 3a) can be accounted for by weak relative upward advection of high values in the subtropics and stronger relative downward advection of low values over the Equator. Below 22 km, slight ascent is inferred over the Equator with poleward motion out to ~20°, followed by vertical divergence. A single plume is seen in QBO easterly shear during October 1988 (Fig. 3b). Lofting is inferred from 20 km to above 28 km. The elevation of the maximum rose by ~4 km over the period of a year, a phenomenon which was probably enhanced by prolonged upward aerosol transport associated with the delayed descent of the easterly shear layer (Fig. 1).

We believe that the primary features of the tropical aerosol distribution are controlled by fluid motions and that aerosol microphysical processes provide only a secondary effect except at the top of the plumes. Because temperature increases upward in the stratosphere, air parcels can ascend only if they experience net radiative heating. Aerosols evaporate as rising parcels warm, or grow in size as sinking parcels cool and water vapour condenses on the droplets. The meridional temperature variation (<5 K) associated with the QBO should cause only a slight enhancement of the observed structure. Theory predicts an extinction ratio variation of less than 10% for a temperature range of 20 K about 220 K at constant altitude with 3.5 p.p.m.v. ambient water vapour²⁰. At the top of a plume, aerosol evaporation is probably more significant than at lower altitudes, and the aerosol distribution there is less useful for inferring the circulation.

In the upper regime, above 20 km, aerosol extinction values are much higher in the tropics than outside. This confirms the early observation⁸ of an aerosol reservoir over the Equator following the eruption of Mount Agung (8° S, 115° E) and is compatible with the maximum in 18°W in the region a year after the 'Hardtack' bomb tests of 1958 (ref. 6). The circulation associated with the QBO tends to spread aerosols around in the tropics, and other mixing processes such as inertial instability²¹

should act to homogenize aerosols in this tropical reservoir. Large gradients in aerosols and PV exist in the subtropics, but the patterns in the two are not identical. Both are in part a manifestation of the dynamics driving the QBO. Another process contributing to these gradients is mixing by planetary Rossby waves, which exist in the extratropical winter hemisphere. These waves propagate freely in westerlies (compare with Fig. 1) and cause quasi-horizontal irreversible mixing as they are absorbed²², yielding enhanced gradients on the edges of the mixing region. Their propagation is controlled by the distribution of PV. Thus it is appropriate to view the subtropical PV gradient as a semipermeable barrier to mixing by extratropical Rossby waves¹⁸. During QBO easterlies, equatorial aerosols are more effectively sequestered from poleward transport processes.

Anthropogenic and natural chemical species entering the stratosphere through the tropical tropopause might be transported poleward relatively rapidly in the lower regime. Trace gases destined for the remainder of the stratosphere would be held temporarily in the upper regime. Detrainment out of the upper reaches of the equatorial reservoir by the Brewer–Dobson circulation would occur more readily during QBO easterly shear, whereas detrainment laterally in the lower regime would occur more readily during QBO westerly shear, preferentially into the winter hemisphere.

A series of eruptions from Mount Pinatubo (18° N, 126° W)

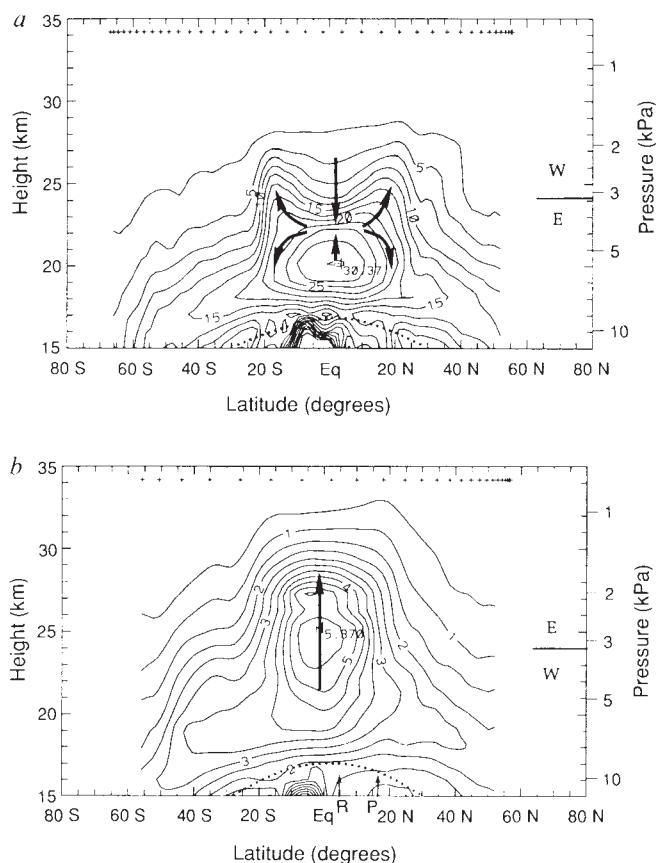


FIG. 3 Latitude-altitude cross-sections of aerosol extinction ratio at 1 μm (refs 13, 14) during two ~40-day periods representative of two different phases of the QBO. *a*, Dominant westerly shear, centred about 11 November 1984, contour interval 2.5; *b*, dominant easterly shear, centred about 4 October 1988, with contour interval 0.5. Crosses indicate locations of the daily average of ~15 profiles. Arrows indicate the inferred QBO circulation based on the aerosol distribution. The altitude of the zero wind over the Equator is transcribed from Fig. 1 at the right of each section. The climatological tropopause is indicated by a dashed line, where cloud tops can contaminate the aerosol data. R and P indicate the latitudes of Mounts Ruiz and Pinatubo, respectively.

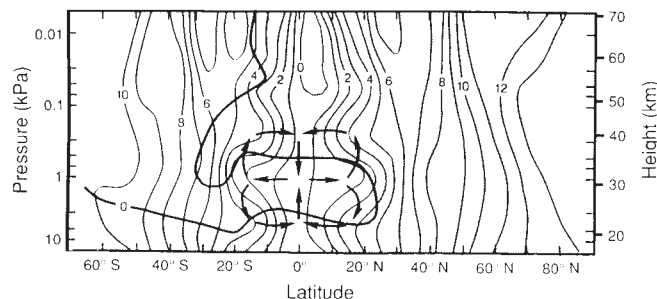


FIG. 2 Latitude-altitude section of normalized zonal mean potential vorticity (PV) derived from satellite temperature data¹⁶ averaged over the period 31 October to 5 November 1978. The zero wind line separates QBO and summer easterlies from westerlies elsewhere. The arrows denote a relative meridional circulation due to the QBO which is compatible with the deformation of PV contours and the relevant theoretical models¹⁰⁻¹². Westerly winds are shaded.

reached the middle stratosphere²³ during June 1991, a time of descending QBO easterly shear (Fig. 1). The wind structure in autumn 1991 similar to that following the eruption of Mount Ruiz (5° N, 75° W) in November 1985 and the structure in November 1988 (Fig. 3b). Recent SAGE II observations and ground-based lidar measurements indicate that Pinatubo material had already spread into the mid-latitudes of the Southern Hemisphere below 28 km and into the mid-latitudes of the Northern Hemisphere below 24 km by September 1991 (P. McCormick, personal communication). We expect that more material will be detrained into the northern mid-latitudes during winter, but as the easterly shear region reaches the lower transport regime, transport towards the poles will be less than if the shear had remained westerly. □

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Thermal disequilibrium at the top of volcanic clouds and its effect on estimates of the column height

Andrew W. Woods* & Stephen Self†

* Institute of Theoretical Geophysics, Department of Applied Mathematics and Theoretical Physics, Silver Street, Cambridge CB3 9EW, UK

† Department of Geology and Geophysics, School of Ocean and Earth Science and Technology, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA

SATELLITE images of large volcanic explosions reveal that the tops of volcanic eruption columns are much colder than the surrounding atmosphere¹. We propose that this effect occurs whenever a mixture of hot volcanic ash and entrained air ascends sufficiently high into a stably stratified atmosphere. Although the mixture is initially very hot, it expands and cools as the ambient pressure decreases. We show that cloud-top undercoolings in excess of 20 °C may develop in clouds that penetrate the stratosphere; this is consistent with observations of the 4 April 1982 eruption of El Chichón¹ and the 18 May 1980 eruption of Mount St Helens². Furthermore, from our model results, we predict that for a given cloud-top temperature, variations in the initial temperature of 100–200 °C may correspond to variations in the column height of 5–10 km. We deduce that the present practice of converting satellite-based measurements of the temperature at the top of volcanic eruption columns to estimates of the column height will produce rather inaccurate results and should therefore be discontinued.

Many of the hazards associated with explosive volcanic eruptions result from the vast clouds of ash which are injected tens of kilometres into the atmosphere. During an eruption, the ash cloud is a danger to aircraft flying near the volcano. During both the eruption of Redoubt, Alaska on 15 December 1989 and the eruption of Pinatubo, Philippines on 15 June 1991, several commercial aircraft flew into ash clouds, leading to temporary in-flight engine failure and considerable damage to the aircraft. So that aircraft can be re-routed, accurate predictions of the location of the ash clouds are required. A key element of any such prediction is knowledge of the eruption column height, particularly because the prevailing winds which disperse the ash often change direction with altitude. Accurate information about the height of rise of the cloud also allows better estimates of both the geographical extent of ash deposits^{3–6} and the long-term climatic impact of an eruption, resulting from sulphuric acid aerosols injected into the stratosphere⁷.

It has been the practice to determine the altitude of volcanic clouds by obtaining cloud-top temperature from a thermal-infrared sensor on a satellite and equating this with the ambient atmospheric temperature obtained from radiosondes on balloon launches^{1,8–10}. But this method has been shown either to give unrealistic cloud altitudes which differ from independent measurements of cloud height or to give cloud temperatures slightly colder than the temperature at any height in the atmosphere^{1,8–11}. Such differences in temperature were not noted to be significant. In several recent large volcanic eruptions (Table 1), however, satellite-perceived cloud-top temperatures have been consistently lower than the temperature of the surrounding atmosphere at that altitude. As far as we are aware, no explanation of this paradox has been proposed. Matson¹ has suggested that his data might be explained by assuming the cloud spreads out laterally at the tropopause, where the ambient temperature has its minimum, even though this minimum may still be a few degrees greater than the cloud-top temperature. We believe that this interpretation is inconsistent with data from the 4 April 1982 'C' phase plume of El Chichón. Images obtained from a GOES satellite, 90 and 120 min after the eruption, show the cloud top had cooled to –82 °C. Independent estimates from numerical models of fallout⁶ and from the wind fields¹ suggest that the cloud ascended to an altitude of 26 ± 3 km. But radio-sonde data from the two nearest Mexican stations, Merida and Veracruz, show the ambient temperatures at altitudes of 26 ± 3 km were in the range –55 to –40 °C. Therefore, at the cloud-top, the ambient temperature was 20–35 °C warmer than the cloud. Indeed, the cloud-top was a few degrees colder than the tropopause (–76 °C) but the tropopause was located at an altitude of 17 km, nearly 10 km below the cloud top.

Our purpose here is to explain and quantify these observations, and thereby predict bounds on the column height as a function of satellite-perceived cloud-top temperatures. The difference in temperature between the relatively cold volcanic cloud tops and the surrounding atmosphere may be understood by considering a simple buoyant plume in a stratified environment¹². Relatively warm fluid, released from a nozzle into a thermally stratified fluid, convects upwards towards the neutral buoyancy height, at which the plume density and hence temperature equal those of the environment. The inertia of the plume fluid then carries it upwards above the neutral buoyancy height, until it has decelerated to rest under gravity. Therefore, at its maximum height, a simple plume is in fact colder than the ambient.

In a volcanic cloud, the situation is more complex. Volcanoes erupt dense, hot mixtures of ash and clasts, at high speed. This mixture entrains ambient air, which is heated and expands, and thereby a buoyant mixture of ash and gas^{13–15} is generated. This buoyant mixture rises into the atmosphere and, as in a simple plume, ascends beyond its neutral buoyancy height where its bulk density equals that of ambient. But a volcanic eruption column contains dense ash particles, and so at the neutral

buoyancy height, the gaseous component of the mixture must be less dense and therefore hotter than the ambient. If the atmosphere was unstratified, with an adiabatic temperature profile, then, as the column entrained ambient, it would cool monotonically towards the ambient temperature. But the atmosphere is stably stratified, and so the ambient temperature relative to the adiabat increases with height. Owing to the decompression and cooling of air entrained at lower altitudes, therefore, the temperature of the eruption column relative to that of a stably stratified ambient decreases more rapidly with height than it would in an unstratified environment. As a result, if the material in the eruption column overshoots the neutral buoyancy height sufficiently, then the cloud-top temperature may fall below the ambient. Our numerical investigations, based on Woods' model¹⁵, suggest that in most plinian eruption columns, the large upward momentum of the column at the neutral buoyancy height causes it to overshoot by several kilometres, which is more than

TABLE 1 Cloud-top undercooling in recent volcanic eruptions

Eruption	Cloud-top temperature* (°C)	Height of cloud† (km)	Ambient temperature at cloud top‡ (°C)	Under-cooling from satellite data (°C)	Model under-cooling§ (°C)
El Chichón, 4 April 1982 (refs 1, 6)	-82	26 ± 3	-55 to -40	20 to 35	20 to 40
Colo, 28 July 1983 (ref. 9)	-79	?	-76	-3	10 to 40
Redoubt, 8 January 1990	-65	> 25	-55 to -45	10 to 20	20 to 50
Pinatubo 15 June 1991	-80	> 30	> -70¶	> 10¶	25 to 50

* Estimated from (GOES) satellite thermal infrared sensors.

† Estimated from independent sources, including models of fallout⁶ and wind data¹.

‡ Estimated from local radiosonde soundings of the atmosphere.

§ Model cloud undercoolings were estimated from the atmospheric structure and uncertainties in the eruption temperature and cloud height.

|| Ambient conditions for the Colo eruption used soundings taken at a considerable distance from the volcano; the only height estimates available are those calculated from the satellite data.

¶ Radiosonde soundings near Pinatubo volcano on 15 June 1991, are only available up to 17 km; estimates from undercoolings taken on other days suggest undercoolings of 30–40 °C.

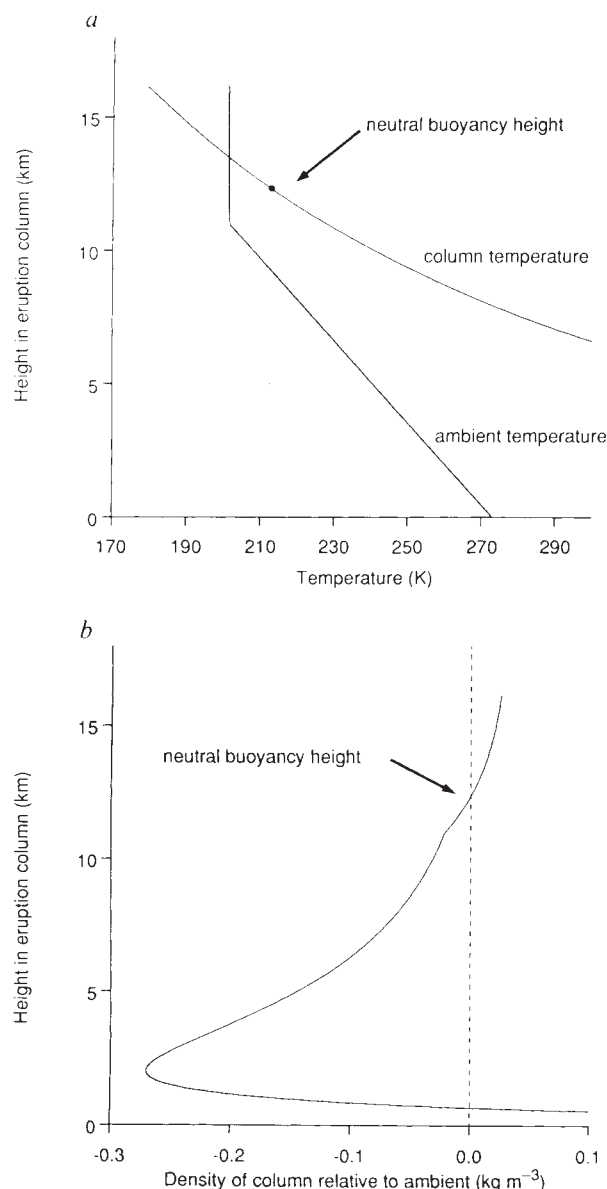


FIG. 1 A typical calculation¹⁵ for an eruption with vent velocity of 100 m s⁻¹, vent radius of 100 m and eruption temperature of 1,000 K showing (a) the variation of the eruption column temperature and ambient temperature as a function of height in the column, and (b) the variation of the density, relative to the ambient, with height in the column. The neutral buoyancy height is marked on each figure.

sufficient to generate undercooling. In Fig. 1, we show that as the material in a typical model column ascends, it remains hotter than the ambient up to, and some distance beyond, the neutral buoyancy height, which is marked with a circle. Above this point, in the upper three kilometres, the column is colder than the ambient. In Table 1, we compare our model predictions of cloud-top undercooling with satellite measurements.

We have used the model to investigate the dependence of cloud-top temperature on column height and eruption temperature¹⁵. In Fig. 2, we show the variation of the cloud-top temperature as a function of column height for three typical eruption temperatures. Below the tropopause, the atmosphere is only weakly stratified, and so its temperature relative to the adiabat increases slowly with height. Therefore the tops of small volcanic columns are only a few degrees colder than ambient. Above the tropopause, the atmosphere is more stably stratified, with the temperature relative to the adiabat increasing much more rapidly with height. As a result, larger columns develop much greater undercoolings as they rise above the neutral buoyancy height and cool through decompression. In the upper stratosphere, the ambient is so stably stratified that the ambient temperature actually increases with height. Therefore, if a large column entrains enough of this upper stratospheric air, the net column temperature may also increase, even though the column becomes very undercooled by the decompression of air which was entrained and has convected upwards from lower in the atmosphere.

Figure 2 also shows that as the eruption temperature increases, the undercooling of the top of the column increases; hotter eruptions generate more buoyancy, and, as a result, they overshoot the neutral buoyancy height further. Relatively small, but hot (1,300 K), eruptions may generate a column with the same cloud-top temperature as a large, but relatively cool (900 K), eruption. But the larger, cooler eruption may inject ash 10–15 km higher into the atmosphere. Therefore, there is no unique relationship between the temperature and height at the top of an eruption column; this is an important result because in many volcanic explosions, the eruption temperature is not known. Our investigations also show that the temperatures at the top of volcanic eruption columns depend on the detailed structure of the atmosphere. The undercooling of a large eruption column of a given height is greater in mid-latitudes than in the tropics, as the tropopause is located much lower in a mid-latitude

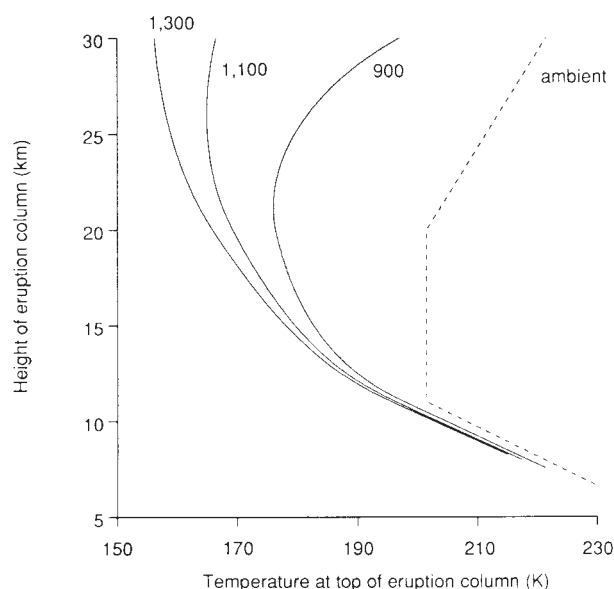


FIG. 2 Model calculations¹⁵ showing the variation of the cloud-top temperature as a function of the height of the eruption column. Curves are given for three eruption temperatures (K). For comparison, the atmospheric temperature profile is also shown (dotted). In these calculations, the eruption velocity was 200 m s^{-1} and the vent radius varied. As the vent radius increases, the mass flux and hence column height increases. Numerical investigation showed that for a given column height and eruption temperature, variations in initial volatile content, eruption velocity and vent radius had only a secondary effect on the cloud top temperature.

atmosphere. Moisture is present in the lower few kilometres of the atmosphere and so may affect the temperatures of smaller columns.

After ascending in the eruption column, ash is carried downwind from the vent as a plume whose temperature gradually adjusts to the ambient. This suggests that satellite measurements of the ash plume temperature far downwind may be compared with radiosonde data to give more reliable estimates of the altitude of the ash. Because the cloud top is relatively dense, the cloud material will tend to fall downwards towards the neutral buoyancy height as it is displaced by new material from below and spreads outwards. This will produce a gravity current intruding into the atmosphere, as observed in the Redoubt eruption cloud on 21 April 1990 and in recent laboratory experiments¹⁶. But owing to fallout of ash and heating of the cloud, the density of the ash plume decreases with distance from the vent; therefore the ash plume may ultimately spread out laterally at an altitude several kilometres above the original neutral buoyancy height of the eruption column.

Preliminary calculations suggest that volcanic thermals, which are generated from short-lived explosions, can develop similar cloud-top undercoolings. This is consistent with the observations of the 18 May 1980 lateral blast cloud at Mount St Helens, which ascended rapidly as a volcanic thermal^{17,18} and which we estimate had a cloud-top undercooling of $\sim 15\text{--}20^\circ\text{C}$.

The top of large volcanic eruption columns may be tens of degrees colder than the surrounding ambient; therefore, there is no simple means of relating satellite-perceived volcanic cloud top temperatures to cloud height by using radiosonde temperature data for the local atmosphere. In conjunction with eruption column models and independent measurements of column height and ambient conditions, however, such cloud-top temperature measurements provide useful constraints on the eruption conditions and the mass of ash injected into the atmosphere. With the advent of long-track stereo imaging and laser

altimetry from space platforms, there may soon be an independent means of obtaining eruption column heights¹⁹. □

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Filtration of colloidal melanin from sea water by planktonic tunicates

Per R. Flood*, Don Deibel† & Claude C. Morris†

* Institute of Anatomy, University of Bergen, N-5009 Bergen, Norway

† Ocean Sciences Centre and Department of Biology, Memorial University of Newfoundland, St John's, Newfoundland A1C 5S7, Canada

PELAGIC tunicates of the genus *Oikopleura* produce, live in and pump water through mucous structures termed 'houses'. Because these houses contain filters with submicrometre pores, oikopleurids have been proposed as important consumers of particulate organic carbon (POC) in the sea¹. We present new evidence that they also consume 'dissolved' organic carbon (DOC) in the colloidal size range down to about $0.2 \mu\text{m}$ in diameter. Such colloids are more abundant in the sea than previously believed^{2,3}, and have an important role in the global flux of carbon. Oikopleurid tunicates have the potential to filter a significant fraction of the water mass every day^{1,4}, and to repack much of the colloidal DOC into their houses, faecal pellets and bodies. Oikopleurids are also known prey for several fish species^{5–7}. Thus, we speculate the oikopleurid tunicates to mediate a substantial energy flow, from DOC through a two-step 'food-chain', to fish of commercial interest, by-passing energy-consuming respiration by bacteria and small planktonic protozoa.

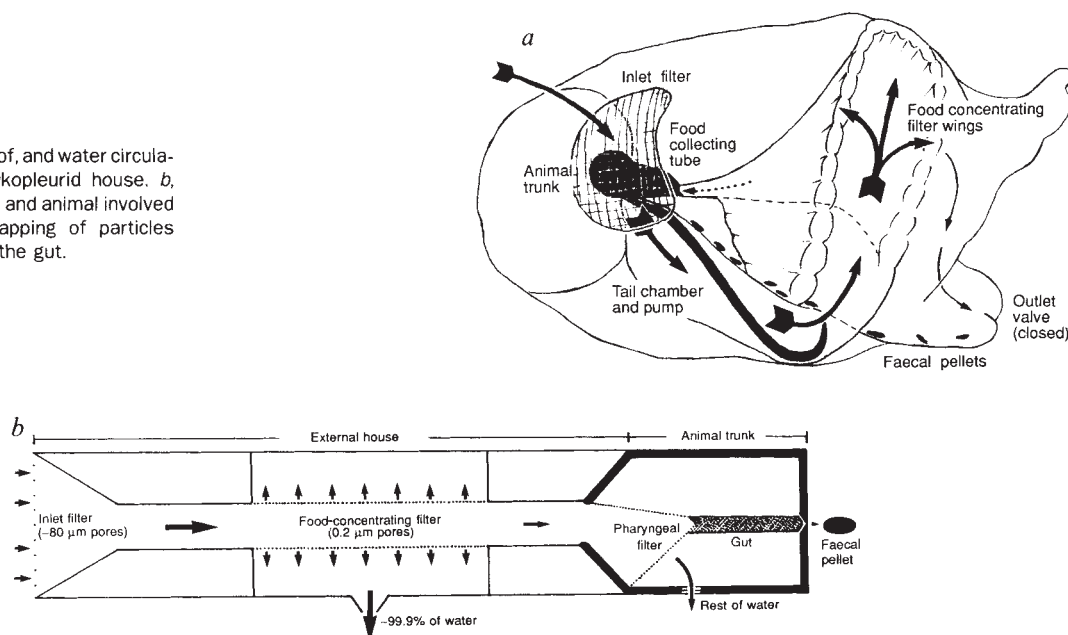
The food-concentrating filters of oikopleurid houses have pores only $0.2 \mu\text{m}$ wide^{8–12}. These filters are used as a tangential flow filtration device to aggregate and concentrate particles suspended in sea water up to 1,000 times¹², before they are ingested by the animal and removed from suspension by an internal pharyngeal filter (Fig. 1)¹³.

There is about $300 \mu\text{M}$ or 3.6 mg l^{-1} DOC in sea water prefiltered through a $0.4 \mu\text{m}$ membrane filter. Most of this DOC is biodegradable polymers of relative molecular mass 4,000–100,000 (ref. 2). Larger colloidal particles, up to $1 \mu\text{m}$ in diameter, account for at least 10% of (additional) DOC³.

To examine whether oikopleurid tunicates are capable of consuming colloidal DOC, we offered suspensions of melanin particles from the ink sac of the cuttlefish *Sepia officinalis* to the common cold ocean tunicate *Oikopleura vanhoeffeni*. This colloid was chosen because of its easy visibility, high stability and lack of tendency to agglutinate¹⁴.

Highly diluted *Sepia* ink was offered to eight adult animals, each in a separate, 450-ml glass jar. Ink, prefiltered according

FIG. 1 *a*, General organization of, and water circulation (arrows) through, the oikopleurid house. *b*, Sequential filters of the house and animal involved in the concentration and trapping of particles before they are conveyed to the gut.



to two operational definitions of DOC¹⁵, was added in concentrations of $0.5\text{--}7\text{ mg l}^{-1}$, covering much of the range of naturally occurring dissolved organic matter in the sea. The jars were not agitated because the ink did not sink or coagulate in control jars without animals over several weeks. The concentration of ink in the jars was measured at intervals by removing 1 ml subsamples for spectrophotometry.

We found that all eight *O. vanhoeffeni* removed *Sepia* ink at constant rates for 1–5 days, in some cases reducing the concentration of ink to <5% of initial values (Fig. 2). Removal was detected after only 1 h, and the ink concentration decreased linearly even when the initial concentration was only $0.5\text{ }\mu\text{g ml}^{-1}$ (Fig. 2a). Clearance rates (see legend to Fig. 2) ranged from 4 to 27 ml h^{-1} , and there was no difference in the mean clearance rate of ink prefiltered through 1.2 versus $0.45\text{-}\mu\text{m}$ pore size filters (*t*-test; d.f. = 6, $P > 0.20$). These clearance rates are only 2–11% of the clearance rate of plastic beads, $10\text{--}20\text{ }\mu\text{m}$ in diameter, determined for animals of similar size¹⁶. Low retention of ink was expected, because its mean ($\pm\text{s.d.}$) particle size was much smaller ($0.13 \pm 0.023\text{ }\mu\text{m}$) than the pore width of the food-concentrating filters ($0.22 \pm 0.04\text{ }\mu\text{m}$)^{10,11}.

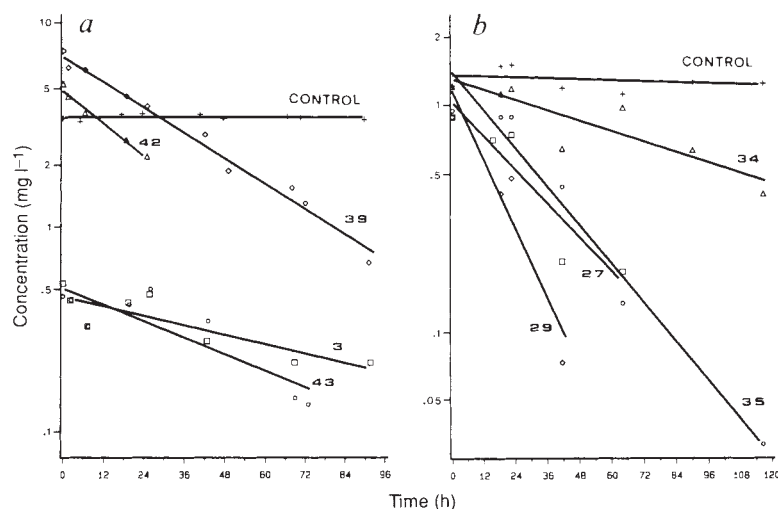
Some of the removed *Sepia* ink adhered to the internal house walls¹⁴, but a large portion of the ink was also ingested by the

animals as evidenced from their blackened pharyngeal filters and gut contents. The diameter of ink particles (mean $\pm\text{s.d.}$) in faecal pellets ($0.17 \pm 0.04\text{ }\mu\text{m}$) was about 2 standard deviations larger than their diameter in ink pelleted by centrifugation (see above, *t*-test; d.f. = 798, $P < 0.001$). Shrinkage of ink particles during processing (perhaps some 30%) does not explain this difference because the samples were treated identically by fixation, plastic embedding and ultrathin sectioning for electron microscopy. The difference rather indicates, by proportions of the standard normal distribution of particle diameter, that the largest 5% of ink particles in suspension were preferentially ingested. This is internally consistent with the retention efficiency of ink versus larger latex microspheres mentioned above.

There was no sign of digestion or cellular uptake of ink particles in accordance with the high stability of naturally occurring melanin biopolymers¹⁷. But using dry milk powder and cell-free filtrates of algal cultures, we have preliminary electron microscopic evidence of ingestion and subsequent digestion of more easily biodegradable colloidal DOC by *O. vanhoeffeni* and *O. dioica*.

It is difficult to relate our results to actual removal rates of colloidal DOC by oikopleurid tunicates in the sea. The size (and shape) distribution of naturally occurring colloidal particles, as

FIG. 2 Removal of colloidal *Sepia* ink from sea water by *O. vanhoeffeni*, measured as the change in absorbance of 500 nm light by a Guilford spectrophotometer. Conversion to dry weight (mg) in volume (l) was done using dilution series made from a known mass of ink. The logarithm of concentration of ink was plotted against time, and the relationship examined for fit to an exponential model, indicating a constant rate of removal of ink from solution. Datapoints and regression lines are presented for each of the eight animals, identified by numbers, and for two jars without animals (control). *a*, Ink prefiltered through a Whatman GF/C glass fibre filter ($1.2\text{-}\mu\text{m}$ nominal pore size). Animal number 42 spawned between 24 and 30 h and died, and animal number 43 had a detached mouth after 72 h, but did not abandon its house. *b*, Ink prefiltered through a $0.45\text{-}\mu\text{m}$ pore size Millipore membrane. Animal number 27 did not build any new houses after 72 h, and animal number 29 reduced the concentration of ink below the detection limits of the technique by 64 h. The slope of the lines is equivalent to the grazing coefficient, which may be converted to clearance rate (ml h^{-1}) by multiplying by the volume of the jar²⁷.



well as their overall quantity as a subfraction of DOC, is only summarily known^{2,3,8,28}. Likewise, we lack precise knowledge about the real straining (and aggregation or agglutination) capacity of oikopleurid food-concentrating filters¹². But even a very conservative estimate of 10% of DOC as grazable by oikopleurids, means that this source of food is as important for the oikopleurids as is total POC. This agrees well with previous findings that POC accounts for a maximum of 30% of the energy needs of *O. dioica*^{19,20}.

Oikopleurid tunicates often occur in high densities in discrete strata at various depths^{21,22}, and may under such conditions clear 30–60% of the water mass in 24 h^{1,4}. Obviously, they may remove and repack colloidal DOC (>0.2 µm particle size) rapidly under such conditions. On the basis of filter parameters, this ability to graze on colloidal DOC is probably shared by caddisfly larvae²³, pedal worms²⁴, ascidians²⁵, salps²⁶ and amphioxus⁹. □

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Sequence identification of 2,375 human brain genes

Mark D. Adams, Mark Dubnick, Anthony R. Kerlavage, Ruben Moreno, Jenny M. Kelley, Teresa R. Utterback, James W. Nagle, Chris Fields & J. Craig Venter*

Receptor Biochemistry and Molecular Biology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892, USA

WE recently described a new approach for the rapid characterization of expressed genes by partial DNA sequencing to generate 'expressed sequence tags'¹. From a set of 600 human brain complementary DNA clones, 348 were informative nuclear-encoded messenger RNAs. We have now partially sequenced 2,672 new, independent cDNA clones isolated from four human brain cDNA libraries to generate 2,375 expressed sequence tags to nuclear-encoded genes. These sequences, together with 348 brain expressed sequence tags from our previous study, comprise more than 2,500 new human genes and 870,769 base pairs of DNA sequence. These data represent an approximate doubling of the number of human genes identified by DNA sequencing and may represent as many as 5% of the genes in the human genome.

Most (83%) of the 2,375 partial cDNA sequences reported here (Table 2) are not related to any previously described sequences. Based on database matches to known genes from humans and from such evolutionarily distant organisms as *Escherichia coli*, yeast, *Caenorhabditis elegans*, *Drosophila*, barley, *Arabidopsis*, rice and green algae, we have putatively identified 217 of the expressed sequence tags (ESTs; Table 1). These include a novel gene similar to *Notch/TAN-1* (refs 1, 2), a new neurotransmitter transporter gene, and a new member of the multidrug resistance gene family. Several genes involved in development or cell differentiation in *Drosophila* are represented by similar human ESTs, including *seven in absentia*³, *big-brain*⁴, the *discs-large* tumour suppressor⁵ and the homeotic gene *orthodenticle*⁶. New members of previously known gene families in humans include a Ca²⁺-transporting ATPase, an ADP ribosylation factor and a new neural-cell adhesion molecule gene.

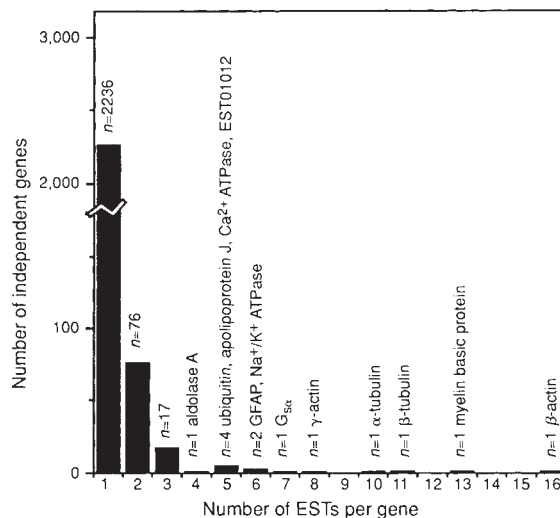


FIG. 1. Redundancy of sequencing of ESTs. The number of putatively identified EST clones plus the groups of ESTs that form contigs are plotted against the number of independent genes represented. The number of genes is given above each bar, with the names of the genes for redundancies of 4–16. We define redundancy as the number of times each gene is represented by an EST; for example β-actin has an EST redundancy of 16. One five-member EST contig was constructed that did not match any known sequences, indicating that at least one common transcript in brain, EST 01012, has not been reported previously. GFAP, glial fibrillary acidic protein.

The 1,971 ESTs without a putative identification were analysed using the coding-region prediction program CRM with the GRAIL server⁷. Some of the unknown ESTs (15%) scored a likely probability of containing protein-coding sequence. Half of the ESTs to known human genes contain protein-coding sequences, so at most half of the unknown ESTs are likely to contain coding sequences. We have found no evidence that genomic DNA or cDNA to unspliced precursor RNA is a major contaminant of either the hippocampus or fetal brain library.

The limited extent of redundancy of EST sequencing is shown in Fig. 1. Of the nuclear-encoded messenger RNAs, the most common ESTs were to the β-actin (0.6% of the EST clones)

* To whom correspondence should be addressed.

TABLE 1 Gene composition of human brain cDNA libraries

EST	Putative Identification	Species	DB	Accession	Length	%ID	EST	Putative Identification	Species	DB	Accession	Length	%ID
EST02243	14.3.3 protein (PK1 regulator)	H	(G)	HUM1433	272	98.9	EST01651	Lactate dehydrogenase A	H	(G)	HUM1434	378	99.5
EST01757	2',3'-cyclic nucleotide phosphodiesterase	H	(G)	HUM1435	272	98.5	EST01652	Lactate dehydrogenase B	H	(G)	HUM1436	401	99.5
EST01784	60K filarial antigen	A	(P)	A28209	88	50.6	EST01764	Lamin B receptor	C	(P)	A36427	76	71.4
EST01455	80K-H protein	H	(G)	HUM19P1A	263	97.3	EST01724	Ion protease	E	(P)	JQ0901	103	41.3
EST01982	ADP-ribosylation factor 1	H	(G)	B33283	84	41.2	EST02413	Long-chain-fatty-acid-CoA ligase	R	(P)	A36275	36	62.2
EST02077	ADP/ATP translocase	H	(G)	HUM1LCA	200	96.5	EST02418	MARCKS homolog	M	(E)	MME52	237	92.4
EST01620	AMP deaminase, brain	R	(P)	A37056	57	100.0	EST01952	MHC class I HLA-Cw2 heavy chain	H	(G)	HUM16CWB	231	97.8
EST01504	Actin, beta, cytoskeletal	H	(G)	HUM1CCYBA	247	98.8	EST01640	MHC class III HSP70-1 gene (HLA)	H	(G)	HUM1HSP	181	100.0
EST01543	Actin, gamma, cytoskeletal	H	(G)	HUM1ACTGM	359	98.9	EST02505	Matrin 3	R	(G)	RATMATRIN3	137	93.5
EST01891	Actinin, alpha	H	(G)	HUM1ACTAR	315	81.6	EST01918	Metalloproteinase inhibitor	H	(G)	HUM1MET	236	99.2
EST01801	Adaptin, beta	H	(G)	HUM1ADPTA	380	99.5	EST01865	Microtubule-associated protein 1B	R	(G)	RATNEDU	293	86.4
EST01710	Adenosine deaminase	H	(G)	HUM1ADAG	166	100.0	EST01473	Microtubule-associated protein 4	H	(G)	HUM1AD4	249	97.6
EST01625	Ala	R	(G)	RATADGR	103	84.6	EST01678	Milk fat globule membrane protein	M	(P)	A36179	48	61.2
EST02113	Alpin	I	(H)	R10002G2_1	92	82.8	EST01704	Monamine oxidase A (MAOA)	H	(G)	HUM1MOA	255	98.8
EST00675	Alcohol dehydrogenase	H	(G)	HUM1ALDH	39	59.0	EST01629	Myelin basic protein	H	(G)	HUM1MBP	314	99.4
EST01660	Aldolase A	H	(G)	HUM1ALDA	346	98.0	EST01580	Myeloid differentiation primary response	M	(H)	MUSK1018_1	76	88.3
EST01761	Aldolase C	H	(G)	HUM1ALDC	317	99.0	EST02585	Myoblast cell surface antigen 24.105	H	(G)	HUM1M2105	177	98.3
EST02688	Alpha-Enolase	H	(G)	HUM1ENOA	345	98.8	EST01614	Myosin heavy chain, non-muscle	H	(G)	HUM1MYCN	291	99.3
EST01635	Alpha-2-Macroglobulin	H	(G)	HUM1MA2M	319	99.4	EST01672	N-formylpeptide receptor	H	(G)	HUM1NFP	237	99.2
EST01664	Amyloid A	H	(P)	A29030	52	54.7	EST01744	NAD(P)+ transhydrogenase (H-specific)	B	(P)	DEBOM	86	93.1
EST01700	Anion exchanger homolog AE3	M	(P)	A33638	95	97.9	EST01338	NF-kappa-B transcription factor	H	(G)	HUM1NFKB	98	98.0
EST01585	Apolipoprotein J	H	(G)	HUM1APOJ	317	99.0	EST01805	Na+/K+ ATPase, alpha subunit	H	(G)	HUM1ATP2	277	99.6
EST01825	Aspartate aminotransferase, cytosolic	H	(G)	HUM1ASAM	309	98.7	EST02610	Neural cell adhesion molecule LI	M	(P)	S05479	82	43.4
EST02671	Aspartate aminotransferase, mitochondria	H	(G)	HUM1ASPAT	231	99.1	EST01771	Neuraxin	R	(P)	S06017	120	84.3
EST01634	Axonal glycoprotein TAG-1	R	(P)	A34695	69	87.1	EST01519	Neurofibromatosis type 1	H	(G)	HUM1NF1	382	98.6
EST02530	B cell-specific Mo-MLV integration site	M	(G)	MUSM11A	111	87.5	EST01749	Neurofilament heavy chain	H	(G)	HUM1NFH	356	99.7
EST02306	Bib protein	D	(P)	S09699	57	53.4	EST02455	Neurofilament subunit M (NF-M)	H	(G)	HUM1NFM	164	99.4
EST01443	CDP-SO-P	E	(P)	JF01668	33	41.2	EST02409	Neutrophil oxidase factor	H	(G)	A34855	43	47.7
EST01800	Ca2+-transporting ATPase	H	(G)	HUM1K1A	380	99.2	EST02632	Modulation protein 1	K	(P)	A24400	63	39.1
EST02146	Calbindin D28	R	(G)	RATCALB28	81	87.8	EST01643	Modulation protein I	K	(P)	A24400	71	50.0
EST01823	Calcineurin A2	H	(G)	HUM1CNAB	287	99.7	EST01961	Notch/Notch homologue	H	(H)	HUM1NLI_1	85	57.0
EST02055	Calcium channel	L	(P)	S05054	33	67.6	EST02429	Nuclear factor I-like protein (NF1)	H	(G)	HUM1NF1A	111	92.0
EST01849	Calmodulin	H	(G)	HUM1CALM	327	98.5	EST01573	Nucleoside diphosphate kinase	H	(P)	A33386	71	52.8
EST01466	Calmodulin-dependent PKI, δ II	R	(P)	A26464	93	98.9	EST01657	Osteonectin/SPARC	H	(G)	HUM1SPARC	348	99.7
EST02378	cAMP-dependent PKI inhibitor	M	(G)	MUSPKI	234	91.5	EST01822	Osteopontin	H	(G)	HUM1OP	170	96.5
EST01644	cAMP-dependent PKI regulatory subunit RIIB	H	(G)	HUM1RIIB	198	97.5	EST01828	Ocd homeotic protein	D	(P)	A35912	35	52.8
EST01628	cAMP-dependent PKI regulatory subunit RIIB	H	(G)	HUM1PKAMD	294	98.3	EST01486	Pancreatic tumor-related protein	H	(G)	HUM1PANCA	422	99.3
EST01041	cAMP-regulated phosphoprotein	B	(P)	B35308	21	86.4	EST01798	Peptidylprolyl isomerase (cyclophilin)	H	(G)	HUM1CYC	382	99.5
EST02447	cAMP-specific phosphodiesterase	H	(G)	HUM1DEFA	363	69.0	EST01751	P-4,5-BPL	R	(P)	A28807	40	90.2
EST01536	Cannabinoid receptor	H	(S)	CANRSHUMAN	97	93.9	EST01656	Phosphoglycerate kinase	H	(G)	HUM1GK1	374	98.7
EST01795	Carboxypeptidase E	H	(G)	X51405	330	99.4	EST02092	Polymyxin B resistance	Y	(P)	A32714	20	76.2
EST01606	Casasein kinase II alpha subunit	H	(G)	HUM1CKII	186	97.3	EST01806	Prohibitin	R	(P)	RATPHOHB	120	97.5
EST01733	Catalase	H	(G)	HUM1CATR	270	96.3	EST01775	Prothymosin cleavage enzyme	M	(H)	MUSPCL1	91	93.5
EST01810	Cathepsin D	H	(G)	HUM1CTD	246	98.4	EST01461	Protein kinase C, beta subunit	H	(G)	HUM1PKCB	155	94.9
EST01799	Cell surface glycoprotein MUC18	H	(G)	HUM1MUC18	413	97.8	EST02087	Protein kinase C, zeta	H	(G)	HUM1PKCZ	382	98.7
EST01487	C/D PG401	H	(G)	HUM1SPG4	261	100.0	EST01650	Protein phosphatase 2A beta subunit	H	(G)	HUM1PP2AB	288	76.8
EST01913	Clathrin coat assembly protein homologue	Y	(H)	YSCYAP54_1	62	63.5	EST01584	PR65 (alpha)	H	(G)	HUM1PR65	242	98.8
EST01796	Coagulation factor VII	H	(G)	HUM1CFVII	227	97.8	EST01786	Protein-tyrosine kinase (clone JTK1)	H	(P)	C38269	38	100.0
EST01676	Cofilin	P	(G)	P1000FIL	132	89.5	EST01572	Protochlorophyllide reductase	W	(P)	S04783	34	57.1
EST01774	Cysteine proteinase inhibitor	H	(G)	HUM1CYSTC	163	97.6	EST01538	Pyruvate dehydrogenase alpha subunit	H	(G)	HUM1PDHA	322	99.7
EST01824	Cysteine-rich intestinal protein	R	(P)	GYR1	56	66.7	EST01587	Pyruvate kinase isozyme M2	H	(G)	HUM1PKM2	395	98.5
EST01502	Cytochrome P-45011E1	H	(G)	HUM1CYP11E	175	97.2	EST02683	Rab1	H	(H)	HUM1RASL1	78	97.5
EST01951	Cytochrome c	H	(G)	HUM1CYCA	172	96.0	EST02515	Rab5	H	(P)	F34323	91	82.6
EST01808	DNA binding protein YAVR267	H	(G)	HUM1YAVR2	429	98.8	EST01072	Rac protein kinase	H	(G)	HUM1RACPC	91	100.0
EST01721	DNA repair helicase (ERCC3)	H	(G)	HUM1ERCC3A	334	98.5	EST01389	Radial spoke protein 3	F	(P)	S05962	58	52.5
EST01621	DNF1552 (lung) mRNA	H	(G)	HUM1DNF1	137	96.4	EST01787	Retinaldehyde-binding protein	H	(G)	HUM1RALBP	129	100.0
EST01257	Diacylglycerol kinase, lymphocyte	P	(P)	S09156	44	42.2	EST01579	Retrovirus-related gag polypeptide	H	(P)	FOH2	95	77.1
EST02477	Diamine acetyltransferase	H	(S)	ATDASHUMAN	74	45.3	EST02050	Retrovirus-related pol polypeptide	H	(G)	GNLGL	50	54.9
EST01508	Dihydrodipicolinate acetyltransferase	H	(G)	HUM1DOCE2	254	98.4	EST01578	Ribosomal protein P0	H	(G)	HUM1RIBP0	289	99.3
EST02062	Dilute (myosin heavy chain)	M	(H)	MUSDILUTE_1	27	100.0	EST01928	Ribosomal phosphoprotein P0	H	(G)	HUM1RIBP0P	273	96.2
EST01719	Disc-large tumor suppressor	H	(G)	DRCLG1_1	53	63.0	EST02158	Ribosomal phosphoprotein P1	H	(G)	HUM1RIBP1	126	91.3
EST02627	Elongation Factor 1 alpha	H	(G)	HUM1EF1A	361	99.2	EST01583	Ribosomal protein L18a	R	(P)	RSTL18	68	35.7
EST01165	Elongation Factor 1 beta	N	(P)	A24806	36	64.9	EST01627	Ribosomal protein L1a	X	(P)	A24579	75	63.1
EST02596	Enolase, gamma-2, neuron specific	H	(G)	HUM1ENOG	345	98.5	EST01667	Ribosomal protein L3	Q	(P)	QJ0771	74	80.0
EST01743	Epoxide hydrolase	H	(G)	HUM1EHD	363	99.7	EST01826	Ribosomal protein S10	S	(P)	R3MY10	36	51.4
EST01946	Ezrin	H	(G)	HUM1EZRN	153	99.3	EST01459	Ribosomal protein Y10	Y	(P)	S11581	40	68.3
EST01971	Familial adenomatous polyposis coli	H	(G)	HUM1FAPC	316	98.1	EST01608	Ribosomal protein S3	H	(G)	HUM1RIBS3	279	95.7
EST01325	Fatty acid synthase	R	(G)	RATFAS	98	79.8	EST02442	Seven in absentia	D	(P)	A36195	46	80.8
EST01476	Fibrillarin	H	(G)	HUM1FBA	201	99.5	EST01960	Spectrin, beta	H	(G)	HUM1SPTB	268	67.7
EST01790	Fibroblast growth factor receptor	H	(G)	HUM1FGFR	376	99.2	EST01699	Sperm membrane protein	R	(P)	A35981	52	58.5
EST01967	Fibronectin	H	(G)	HUM1FNCT	120	98.3	EST01760	Spermidine/spermine N1-acetyltransferase	H	(G)	HUM1SPMAT	102	97.1
EST02186	Fodrin, alpha	H	(G)	HUM1FODX	282	98.2	EST01545	Sphingomyelin phosphodiesterase	H	(G)	HUM1SMPL	159	97.5
EST02428	Fumarate hydratase, mitochondrial	H	(G)	HUM1FHA	96	96.9	EST01984	Stathmin (p18)	H	(G)	HUM1STH	180	100.0
EST01665	G(i) alpha	H	(G)	HUM1G1A	310	98.1	EST01697	Succinate dehydrogenase flavoprotein	B	(H)	BOVSDHFP1_1	44	100.0
EST02389	G(i) alpha	H	(G)	HUM1G1A	275	99.3	EST00472	Synaptotagmin (p65)	H	(H)	SV65HUMAN	27	53.6
EST02362	GAB binding protein, beta subunit	M	(H)	MUSGAB1	86	90.8	EST01586	T-cell surface glycoprotein E2	H	(G)	IL16996	277	99.6
EST01745	GTP-binding protein beta chain homologue	H	(G)	HUM1GABL23	319	99.1	EST01809	TAP-1, 26-kDa cell surface protein	H	(G)	HUM1TAP1	271	96.5
EST00825	Gamma-aminobutyric acid transporter	R	(P)	A35918	26	59.3	EST01955	TCTE1	H	(G)	HUM1TCTE1	62	98.8
EST01738	Gelation factor ANP-280	H	(P)	A37098	74	80.0	EST01575	TFEB transcription factor	M	(H)	MUSTALNR_1	79	81.2
EST01776	Gelsolin precursor, plasma	H	(G)	HUM1GELS	171	99.4	EST02402	Talin	B	(P)	ROBO	65	81.8
EST01649	Glial fibrillary acidic protein	H	(G)	HUM1GFAP	399	99.2	EST01601	Thiosulfate sulfurtransferase (rhodanese)	H	(G)	HUM1RSTNT	228	99.6
EST01965	Globin, gamma	H	(G)	HUM1GLG1	147	100.0	EST01435	Threonyl-tRNA synthetase	H	(G)	HUM1GSA	58	98.3
EST02192	Glutamate decarboxylase	H	(G)	HUM1GAD	213	100.0	EST02420	Thrombospondin precursor	H	(G)	HUM1THY1A	353	98.6
EST01702	Glutamate dehydrogenase	H	(G)	HUM1GHD	223	96.4	EST01783	Thy-1 glycoprotein	H	(G)	HUM1THY10	88	97.8
EST02446	Glutamate-aspartate carrier protein	E	(P)	JVO092	57	37.9	EST02325	Thymosin beta-10	H	(G)	HUM1THY10	287	99.3
EST02034	Glutaminase	T	(S)	GLSRRAT	34	74.3	EST02451	Transcription factor ETR101	H	(G)	HUM1ETR101	167	99.3
EST01576	Glyceroldehyde-3-phosphate dehydrogenase	H	(G)	HUM1GPD	428	99.8	EST01705	Transducin beta-2 subunit	H	(G)	HUM1TRNB	126	100.0
EST01621	HLA-B-associated transcript 2 (BAT2)	H	(G)	HUM1BAT2	301	98.7	EST02047	Triose-phosphate isomerase	H	(G)	HUM1TPI	258	98.8
EST01462	Heat shock cognate protein 70	H	(G)	HUM1HSC70	290	97.2	EST01512	Tubulin, alpha	H	(G)	HUM1TUBA	223	75.0
EST01637	Heat shock protein 90	H	(G)	HUM1HSP90	265	99.6	EST01490	Tubulin, beta	H	(G)	HUM1TUBB	298	93.6
EST01610	Heparin-binding growth factor 1	H	(G)	X51913	235	94.5	EST01530	Tubulin, beta	H	(G)	HUM1TUBB	212	99.1
EST02315	Hesokinase 1	H	(P)	A31969	107	96.3	EST02169	Tyrosine kinase	H	(G)	HUM1HCK	384	74.3
EST01709	High density lipoprotein binding protein	H	(G)	HUM1HBP	282	99.6	EST01557	Ubiquitin	H	(G)	HUM1UBQ	329	97.3
EST01639	Histone H1	M	(P)	A37779	63	75.0	EST01769	Ubiquitin activating enzyme E1	H	(G)	HUM1UBQA	362	91.2
EST02556	Histone H1 (G)	H	(E)	HSHTS10G	172	97.7	EST00853	Unc-104 (kinesin homologue)	J	(P)	JUN0114	37	45.5
EST02528	hRNAP core protein A1	H	(G)	HUM1HNRPA	151	84.9	EST01687	Uroporphyrinogen decarboxylase	H	(G)	HUM1UROD	297	99.3
EST02533	Hypothetical 43.5K protein	M	(P)	JU0319	43	52.3	EST02557	V					

TABLE 2 Gene composition of human brain cDNA libraries

	Total	Hippocampus unscreened	Hippocampus prescreened	Fetal brain*	Fetal brain†	Whole brain‡
No database match	1,942	474	394	1,025	32	17
Exact human match	255	76	88	87	0	4
Non-exact human match	51	8	10	33	0	0
Non-human match	99	34	26	35	2	2
Alu repeat	313	70	70	172	1	0
L1 repeat	58	13	5	39	0	1
THE-ltr sequence	17	1	1	14	1	0
Other repeat	9	3	4	2	0	0
Mitochondrial	181	115	33	27	0	6
rRNA	57	29	7	16	5	0
poly(A) insert only	339	171	161	5	0	2
Total	3,321	994	799	1,455	41	32

Four cDNA libraries were used as sources of clones for sequencing. Human hippocampus and fetal brain (*) libraries, plasmid template preparation, sequencing reactions, and automated sequencing were performed as described¹. A pooled probe consisting of inserts from 10 different EST clones with sequences that matched either mitochondrial genes or the 18S or 28S rRNAs was used to prescreen a gridded filter array of the hippocampus library; nonhybridizing clones are referred to as the 'prescreened library'. Another fetal brain library (†) was constructed by and was a gift from B. Soares (Columbia University). A directionally-cloned library (‡) was prepared¹⁴ using human adult brain mRNA from Clontech (Palo Alto). Of 482 clones analysed by restriction-enzyme digestion, 33% contained inserts at least 1,500 base pairs long. Stratagene hippocampus and fetal brain library totals include data from ref. 1. Sequences of nuclear-encoded cDNAs that did not include the interspersed repeats Alu, L1 or THE-ltr¹⁵⁻¹⁷ were searched against GenBank and, in 6-frame translation, against a comprehensive, non-redundant peptide database using the network BLAST¹⁸ server at the National Center for Biotechnology Information. For significant similarities, a putative gene name and protein identification resource (PIR) gene family identification¹⁹ for the EST were assigned. ESTs without significant matches using BLAST were searched in translation against PIR using the program FASTA. Ten additional marginal matches were found. A total of 2,300 new EST sequences comprising 765,505 nucleotides from the current data set have been submitted to GenBank and assigned accession numbers M77851-M79278 and M85308-M86179. All ESTs except those multiply representing actin, tubulin, and myelin basic protein clones were submitted. cDNA clones from which ESTs were derived are available from the American Type Culture Collection (Rockville, Maryland) with accession numbers 77501-78999 and 81000-81756. The Genome Data Base²⁰ expressed D-segment numbers for these clones are DOS1E-DOS2300E. We have developed a database which includes the clone and sequence data, sequence analysis results, physical mapping data, tissue localization and cross-references to the public databases and distribution of the clones, mapping and sequence data using the Sybase relational database management system (Sybase Inc., Emeryville, California). Comprehensive reports on the sequences described in this paper are available in electronic form. A README file describing how to access the EST database reports is available via anonymous file transfer protocol (FTP) to briggs.ninds.nih.gov. Questions on data access and database structure can be addressed via electronic mail to arkerlav@briggs.ninds.nih.gov.

and myelin basic protein genes (0.5% of the clones). Myelin basic protein, a highly expressed structural component of nerve tissue⁸, displays four alternate splicing forms, of which at least two are present among the ESTs reported here. Other common ESTs were G-protein subunit G_{sα}, γ-actin and both α- and β-tubulin.

All of the genes for which four or more ESTs were found have been sequenced in humans, except for one which was matched by five unknown ESTs. Assuming that most brain mRNAs are rare transcripts⁹, the chance of finding a new gene by EST sequencing is fairly high when ribosomal and mitochondrial transcripts are eliminated. Therefore, although normalization may be important as we near closure in sequencing every human gene, it is not necessary at this stage to reduce sequencing redundancy or to increase gene diversity. Furthermore, a certain amount of redundancy is desirable to the extent that it promotes assembly of EST contigs into full-length cDNA sequences.

By matching ESTs to known database sequences, a phenotypic characterization of the tissue begins to emerge. Protein super-families matched by ESTs were grouped into three broad functional categories to assess the biological spectrum represented

by these randomly selected cDNA clones. Structural and metabolic classes comprised about 30% of the ESTs each, 25% were involved in regulatory pathways and the remainder were not classifiable. Eleven of the eighteen enzymes of glycolysis and the citric acid cycle are represented by at least one subunit or isozyme. In addition, several genes not previously known to be expressed in the brain were matched, including spermine/spermidine acetyltransferase¹⁰ and osteopontin¹¹. Isolation of 171 ESTs from mouse testes was recently reported¹², including four with database matches in common with human ESTs.

The genomic mapping of these new human expressed genes is among our highest priorities. Physical mapping of the 2,375 EST clones reported here would provide human chromosome markers spaced an average of 1.2 megabases apart and would roughly double the number of expressed sequences that have been localized to chromosomes¹³. Mapped ESTs are a new resource for identifying candidate genes for the estimated 5,000 single-locus diseases¹³. All the sequences and clones described here are publicly available (Table 2). We shall update EST clone identification and map information through the NIH cDNA database. □

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Waardenburg's syndrome patients have mutations in the human homologue of the *Pax-3* paired box gene

Mayada Tassabehji*, Andrew P. Read*†, Valerie E. Newton‡, Rodney Harris*, Rudi Balling§, Peter Gruss|| & Tom Strachan*

* University Department of Medical Genetics, St Mary's Hospital, Manchester M13 0JH, UK

‡ Centre for Audiology, University of Manchester, Oxford Road, Manchester M13 9PL, UK

§ Max Planck Institute for Immunobiology, D-7800 Freiburg, Germany

|| Max Planck Institute for Biophysical Chemistry, D-3400 Göttingen, Germany

WAARDENBURG'S syndrome (WS) is an autosomal dominant combination of deafness and pigmentary disturbances, probably caused by defective function of the embryonic neural crest^{1,2}. We have mapped one gene for WS to the distal part of chromosome 2 (ref. 3). On the basis of their homologous chromosomal location, their close linkage to an alkaline phosphatase gene, and their related phenotype, we suggested that WS and the mouse mutant *Spotch*^{4,5} might be homologous. *Spotch* is caused by mutation in the mouse *Pax-3* gene^{6,7}. This gene is one of a family of eight Pax genes known in mice which are involved in regulating embryonic development; each contains a highly conserved transcription control sequence, the paired box⁸. Here we show that some families with WS have mutations in the human homologue⁹ of *Pax-3*. Mutations in a related gene, *Pax-6*, which, like *Pax-3*, has both a paired box and a paired-type homeobox sequence, cause the *Small-eye* mutation in mice¹⁰ and aniridia in man¹¹. Thus mutations in the Pax genes are important causes of human developmental defects.

To identify patient-specific mutations in the human *Pax-3* gene, we designed primers to amplify exons of the gene *HuP2* (ref. 9). *HuP2* was isolated by screening a human λ phage library with a *Drosophila* paired-box probe, and shares extensive sequence homology and organization with the mouse *Pax-3* gene⁸. The *HuP2* sequence⁹ contains three exons, which we designate exons 2, 3 and 4 because of their correspondence with exons 2, 3 and 4 of the mouse *Pax-3* gene. When the amplified exons were tested for heteroduplex formation on Hydrolink polyacrylamide gels¹², variant bands were seen in 6 out of 17 unrelated WS patients. Three of the variants were seen in exon 2, two in exon 3 and one in exon 4. No variants were seen in any exon in 50 normal controls. Thus 6 out of 17 chromosomes carrying Waardenburg's syndrome, but 0 out of 130 normal chromosomes (two each from the fifty controls, plus thirty from the WS patients and families), showed a variant band on the Hydrolink gels ($P = 1 \times 10^{-6}$, Fisher's exact test, one-tailed).

In three of the families the heteroduplex analysis has been extended to all family members. Of the other three variants, two are familial and one is apparently *de novo*, but the whole families have not yet been tested. In the three pedigrees studied, the variant bands show perfect linkage to WS (Fig. 1). These families had all previously been studied for linkage between WS and *ALPP*, the placental alkaline phosphatase marker with which we first localized WS to distal 2q (ref. 3). Families WS.05 and WS.06 had shown no recombinants; family WS.11 had one *WS-ALPP* recombinant (see Fig. 1 of ref. 3), but it is non-recombinant for *WS-Pax-3*. Thus independently of the mouse evidence, *Pax-3* is a candidate gene for WS both by association and linkage.

Amplified mutant alleles from family WS.05 were cloned and sequenced, revealing an 18-base-pair (bp) deletion in the central region of exon 2. This results in loss of amino acids 29 to 34 of the paired domain (Fig. 2). The deleted sequence runs between two directly repeated GGCCC sequences (underlined in Fig. 2c). From the appearance of the heteroduplexes, family WS.06 has a small change (point mutation or very small deletion) in exon 2 and family WS.11 has a deletion in exon 4. Studies of these variants are in progress.

The deletion in family WS.05 affects a highly conserved sequence in the paired domain: the *HuP2* sequence shows 92% nucleotide sequence homology and 100% amino-acid sequence homology with the mouse *Pax-3* sequence over amino acids

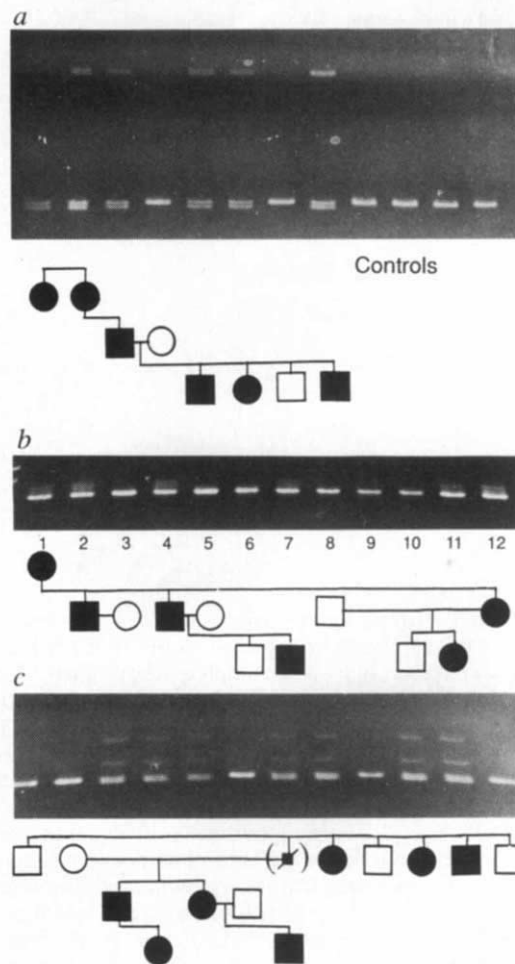


FIG. 1 Heteroduplex analysis of mutations in *HuP2* in Waardenburg's syndrome families. **a**, Exon 2 in family WS.05. Affected family members show a doublet (normal and mutant homoduplexes) plus a slow-moving heteroduplex band. Four right-hand lanes, control samples. **b**, Exon 2 in family WS.06. Affected family members show a close triplet. Lane 12 contains the same amplified DNA as lane 9, but at higher concentration. **c**, Exon 4 in family WS.11. A deceased affected spouse (from whom we have no DNA) is shown in the pedigree to make clear the family relationships. Affected family members show a tight, hardly resolved, doublet plus two faint slow-moving heteroduplex bands.

METHODS. Genomic DNA samples were amplified by polymerase chain reaction (PCR) essentially as before¹⁸ using *HuP2*-specific primers as follows: exon 2, 5'-GAAGACTGCGAAATTACGTGTCG-3' and 5'-GACCACAGT-CTGGAGCCAGGAGG-3'; and exon 4, 5'-AGCCCTGCTGTCTCAACCATGTG-3' and 5'-TGCCCTCCAAGTACCCAGCAAGT-3'. After amplification, the PCR product was heated at 95 °C for 5 min, cooled in ice for 2.5 min then held at 20 °C for 60 min to allow heteroduplex formation¹². Samples (10 μ l) of the 100- μ l PCR products were loaded onto D-5000 Hydrolink gels (AT Biochem)¹² and electrophoresed overnight at 30 V.

† To whom correspondence should be addressed.

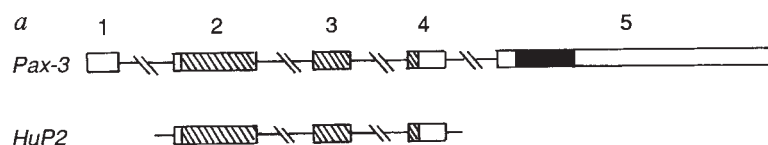
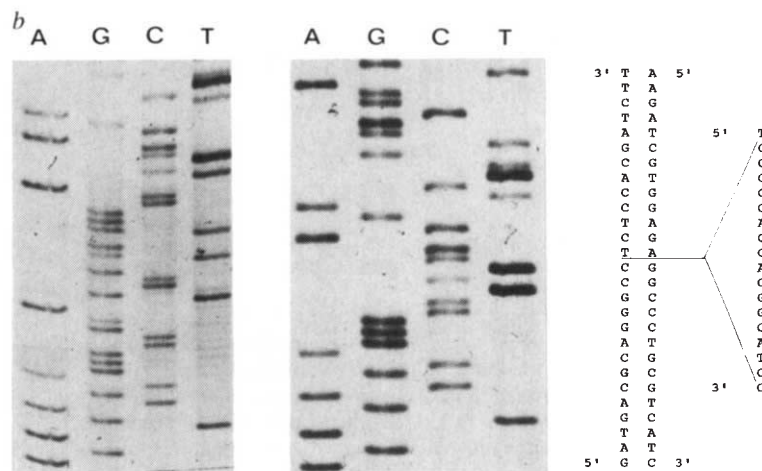


FIG. 2 Sequence analysis. *a*, Homology between *Pax-3* and *HuP2*. Boxes indicate characterized exons; striped areas mark the paired box and filled area marks the paired-type homeobox. *b*, DNA sequence of normal and mutant exon 2 in family WS.05. Left-hand panel, normal sequence; right-hand panel, mutant sequence. *c*, Partial sequence of protein products of normal and mutant genes.

METHODS. A 150-bp *HincII*-*KpnI* fragment of the amplified exon 2 was resolved into mutant and normal bands on a 3% agarose gel. The fast-moving mutant band was excised, and the DNA was purified and cloned into pTZ18R (Pharmacia) which had been digested with *HincII* and *KpnI*. Dideoxy DNA sequencing was conducted on single-stranded recombinants using the Sequenase system (USB) with a reverse M13 sequencing primer.



c

WS.05 MUTATION (exon 2)

	Arg	His	Lys	Ile	Val	Glu	Met	Ala	His	His	Gly	Ile	Arg	Pro	Cys	Val	Ile	Ser	Arg
NORMAL	CGC	CAC	AAG	ATC	GTG	GAG	ATG	GCC	CAC	CAC	GGC	ATC	CGG	CCC	TGC	GTC	ATC	TCG	CGC
MUTANT	CGC	CAC	AAG	ATC	GTG	GAG	A---	---	---	---	---	---	---	---	---	---	---	---	---
	Arg	His	Lys	Ile	Val	Glu	A(												

2-121. The loss of six amino acids in this region in the deletion in family WS.05 would be expected to be clinically significant and may conceivably lead to complete loss of *Pax-3* function. In agreement with this, a recently identified mouse *Pax-3* mutation that causes the *Splotch* phenotype⁷ truncates the protein in the homeobox. The deletion in WS.05 does not alter the translational reading frame, however, so it remains possible that a mutant *Pax-3* product may have a dominant effect.

Clinically Waardenburg's syndrome is divided into type 1 and type 2 (ref. 2). Both forms are autosomal dominant, but type 1 has a characteristic facial feature, dystopia canthorum (outward displacement of the inner canthi of the eyes), which is absent in type 2. It has been shown (L. Farrer *et al.*, manuscript submitted) that in some families Waardenburg's syndrome (type 1 or type 2) is not linked to *ALPP*. This genetic heterogeneity is not surprising because in the mouse many non-allelic mutations produce the phenotype of deafness and white spotting^{13,14}. The three families studied here all have type 1 WS. Clinical features of affected members have been listed³. Briefly, all affected people have dystopia canthorum and varying combinations of heterochromia irides, deafness, white forelock and other abnormal pigmentation. All meet the diagnostic criteria for Waardenburg's syndrome type 1 agreed by the Waardenburg Mapping Consortium (L. Farrer *et al.*, manuscript submitted).

We conclude that the *Pax-3* mutations are the cause of the Waardenburg's syndrome in these families. Thus *Pax-3* is one of the genes that mutates to give Waardenburg's syndrome, and Waardenburg's syndrome and *Splotch* are homologous, as we originally suggested. Identification of *Pax-3* as the gene responsible for *ALPP*-linked Waardenburg's syndrome opens the way to understanding the molecular pathology of this disease.

The way in which the Pax genes regulate embryonic develop-

ment is unknown. Pax genes were initially isolated on the basis of their sequence similarity to *Drosophila* segmentation and segment polarity genes such as *paired* and *gooseberry*^{15,16}. Subsequent investigations have shown that Pax gene products are DNA-binding proteins and probably act as transcription factors^{6,17}. All contain the paired box, and *Pax-3* and *Pax-6* also contain a paired-type homeobox. So far, our search for mutations has covered only three exons of the human *Pax-3* gene. By analogy with the mouse *Pax-3* gene, there should be two further exons, one beyond each end of the published *HuP2* sequence, and exon 5 should contain a paired-type homeobox. If the *ALPP*-linked WS gene has a paired box and a homeodomain, the molecular pathology will be very interesting. □

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An exonic mutation in the *HuP2* paired domain gene causes Waardenburg's syndrome

Clinton T. Baldwin*, Christopher F. Hoth*,
Jean A. Amos*, Elias O. da-Silva†
& Aubrey Milunsky*†§

* Center for Human Genetics and † Department of Pediatrics,
Boston University School of Medicine, 80 East Concord Street,
Boston, Massachusetts 02118, USA

‡ Instituto Materno-Infantil de Pernambuco and Department of Genetics,
Federal University of Pernambuco, Recife, PE, Brazil

HERE we report the identification and characterization of a gene defect causing Waardenburg's syndrome with hearing loss in a large Brazilian family. This demonstrates a mutation causing Waardenburg's syndrome as well as a mutation causing a form of congenital deafness. The mutation was found in the *HuP2* gene, a member of the paired domain family of proteins that bind DNA and regulate gene expression¹. The mutation occurred in 100% of the cases with the disease in this family and was absent in a random sample of 50 unrelated control subjects. Identification of the Waardenburg's syndrome gene and future characterization of its gene product is likely to increase our understanding of the pathogenesis of this disorder and may allow prevention of deafness of this type.

The clinical phenotype of the family analysed here was fully described elsewhere (ref. 2, kindred 1) and consists of >100 individuals in six generations, of which 49 have a diagnosis of Waardenburg's syndrome type I (WSI). The major diagnostic criteria are sensorineural deafness, pigmentary abnormalities of the iris, hypopigmentation of hair (for example, white forelock) and dystopia canthorum^{3,4}. In this family, telecanthus is the only constant anomaly in all the affected individuals. The family is unusual in that >78% of the affected members have noticeable hearing loss.

Attention to a potential chromosomal location for WSI was focused on 2q35-2q37 after a description of this disorder in an affected Japanese child with a paracentric inversion and an apparent *de novo* mutation⁵. Subsequent linkage analysis^{6,7} confirmed this locus. Several other lines of evidence have implicated the human *HuP2* paired domain gene at 2q35-2q37 as a candidate gene for WSI. The *HuP2* protein is a member of the paired domain family of proteins that, like homeodomain proteins, binds DNA and regulates gene expression⁸⁻¹⁰. A mutation in *Pax3*, the murine homologue of *HuP2*, causes the Splotch (Sp) phenotype in mice¹¹. When homozygous, the Splotch phenotype includes exencephaly, spina bifida, dysgenesis of neural crest cell-derived spinal ganglia and defects in other structures derived from neural crest cells. Heterozygous Splotch mice, like WSI, have facial defects, pigmentary disturbances, and hearing loss.

The gene structure near the three exons encoding the paired domain of the *HuP2* gene has been described¹. To screen for mutation, oligonucleotide primers corresponding to sequences flanking the exons were used in a polymerase chain reaction (PCR) (Fig. 1) and the amplification product analysed by single-strand conformation polymorphism¹². The results (Fig. 2) for exon 2 of *HuP2* demonstrate a complex pattern of at least six bands. One band (shown by arrow in Fig. 2) is present in all affected members of the family but is absent in all the unaffected members or control subjects. The remaining five bands failed to cosegregate with the disease.

The single-strand conformation polymorphism analysis suggested a sequence variation in exon 2 of *HuP2* in affected

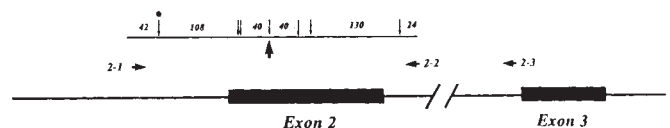


FIG. 1 A partial gene map of the human *HuP2* gene. Primers 2-1 (5'-TGTCGAGCAGTTTCAGCG), 2-2 (5'-CAGTCTGGGAGCCAGGAG) and 2-3 (5'-CGTTGGTACCCGGTACCC) were used to amplify exon 2 of the *HuP2* gene using PCR²⁰. Primer 2-1 corresponded to bases 164-182, primer 2-2 to bases 555-573 and primer 2-3 to bases 1,813-1,831 (ref. 1). The position of exons in the amplification product is shown. Exon 2 is presumed to be the second exon of *HuP2* by analogy with *Pax3* (ref. 13); however, the sequence of the first exon of *HuP2* has not been described. The position of the *HaeIII* sites (down arrows) and predicted fragment sizes after *HaeIII* digestion of the amplification product (2-1 to 2-2) is shown in the upper segment. The site of mutation is shown by an up arrow. The *HaeIII* site with a circle indicates the polymorphic *HaeIII* sites in an intron.

members of the family. To characterize this variation, the amplification product of this exon was sequenced directly (Fig. 3). The sequences of exon 2 of *HuP2* from control subjects were identical to those previously reported¹. But the amplification product from affected family members contained two bands (G/A) 64 bases downstream from the 5' end of exon 2. The G band at this position corresponds to the normal sequence in the antisense strand, whereas the unusual A residue substitutes the second base of a Pro codon (CCG) making it a codon for a Leu residue (CTG) in the sense strand.

This single-base substitution at this position destroys a *HaeIII* restriction site in *HuP2* exon 2. Thus, we tested all 60 available members of the Brazilian family, 50 unrelated control samples and 17 other unrelated WSI families for the absence of this restriction site (Fig. 4). All 26 affected members of the family were heterozygous for the *HaeIII* site, whereas all 34 unaffected members and 50 control subjects were homozygous for the presence of the *HaeIII* site. We did not detect the Pro to Leu mutation in the 17 unrelated Waardenburg's syndrome families. The results of the *HaeIII* digestion also identified a polymorphic *HaeIII* site located in the intron preceding exon 2 (Fig. 4). But the polymorphism did not coinhere with WSI and was present in control subjects.

HuP2 and its murine homologue *Pax3* belong to the paired domain family of proteins that contain a helix-turn-helix

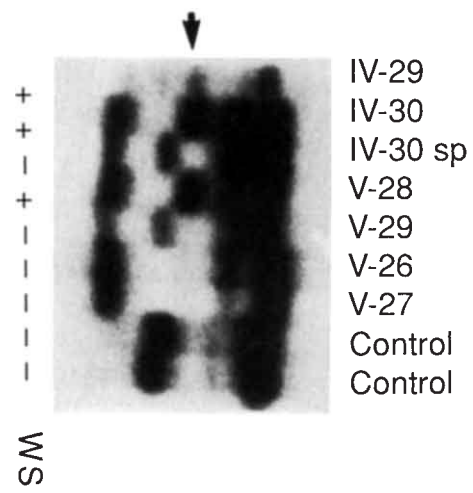


FIG. 2 Single-strand conformation polymorphism (SSCP) analysis of *HuP2* exon 2. Primers 2-1 and 2-2 (Fig. 1) were used to amplify *HuP2* exon 2 from several members of the Brazilian WSI family. Individuals are numbered as previously described². The amplification product was then analysed by SSCP as previously described¹². The arrow indicates the band present in affected family members and absent in unaffected and control subjects. Plus sign, affected; minus sign, unaffected by Waardenburg's syndrome.

§ To whom correspondence should be addressed.

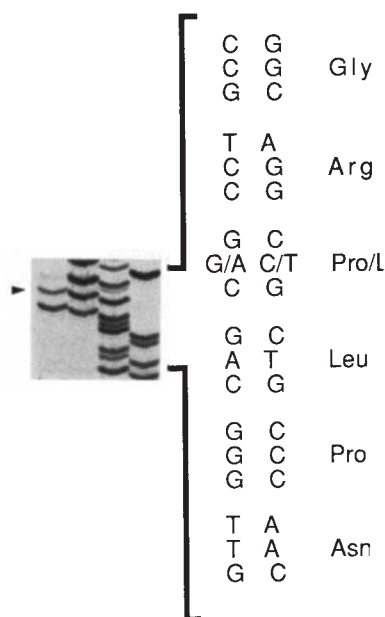


FIG. 3 DNA sequencing gel of a Pro/Leu heterozygote. Primers 2-1 and 2-3 (Fig. 1) were used to amplify HuP2 exon 2 from affected and unaffected (not shown) members of the Brazilian family. The amplification product was sequenced by two PCR reactions. In the first amplification using genomic DNA as template, 5 pM primer 2-1 and 5 pM of primer 2-3 were used in a 100- μ l reaction volume²⁰. A sample (1 μ l) of this reaction was used as template in a second amplification using 50 pM primer 2-1 and 1 pM primer 2-3 in a 100 μ l reaction volume. The product was then sequenced with primer 2-2 by the dideoxy method as previously described^{21,22}.

structural motif which binds DNA^{1,8,13}. The missense mutation reported here replaces a Pro residue with a strongly hydrophobic Leu residue in a region just preceding one of the helices of the paired domain structure⁸. This Pro residue in this position is invariant in paired domain proteins¹ and seems to be important in determining the specificity of DNA binding. A mutation (Gly to Ser) in the *Pax 1* paired domain gene only three amino acids upstream from this Pro residue causes the mouse mutant

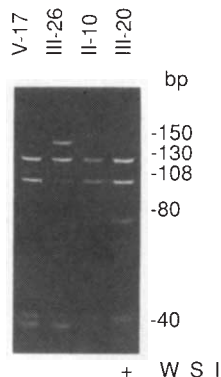


FIG. 4 *Hae*III digestion of PCR-amplified DNA. Primers 2-1 and 2-2 (Fig. 1) were used to amplify the HuP2 exon 2 from each of several members of the Brazilian WSI family. Individuals are numbered as previously described². The presence of the mutation destroys an *Hae*III site between two 40-base-pair (bp) fragments. Thus the presence of two 40-bp fragments and an 80-bp fragment is characteristic of a Pro/Leu heterozygote, whereas the presence of only 40-bp fragments is characteristic of a Pro/Pro homozygote. Results of *Hae*III digestion also identified a second polymorphic *Hae*III site between the 108- and 42-bp fragments. Thus, a 150-bp fragment is also present in some samples. Plus sign, affected; minus sign, unaffected by Waardenburg's syndrome.

phenotype undulated¹⁴. This mutation reduced the ability of *Pax 1* to bind target DNA by over 10-fold and created affinity with DNA sequences not present in normal *Pax 1* (ref. 15).

Affected members of the Brazilian family are the only individuals containing the Pro to Leu mutation. Thus it is likely that this mutation is exclusive to this pedigree, as is the case for many disorders such as osteogenesis imperfecta¹⁶, cystic fibrosis¹⁷ and neurofibromatosis¹⁸. Interestingly, 78% of the Brazilian family demonstrate noticeable hearing loss which is usually present in only 20% of the Waardenburg's syndrome cases². Thus, a mutation in this region of the *HuP2* protein may have an especially important effect on the development of hearing. Studies of the domestic white cat¹⁹, an animal model for Waardenburg's syndrome, revealed that they have unilateral or bilateral deafness as in humans. The authors concluded that, in the domestic white cat destruction of the inner ear mechanism occurs in the first days after birth, and is correlated with a failure to regulate the constitution of the endolymphatic fluid¹⁹. □

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Rapid regeneration of the actin-myosin power stroke in contracting muscle

Vincenzo Lombardi, Gabriella Piazzesi & Marco Linari

Dipartimento di Scienze Fisiologiche, Università degli Studi di Firenze, Viale G. B. Morgagni 63, I-50134 Firenze, Italy

AT the molecular level, muscle contraction is the result of cyclic interaction between myosin crossbridges, which extend from the thick filament, and the thin filament, which consists mainly of actin. The energy for work done by a single crossbridge during a cycle of attachment, generation of force, shortening and detachment is believed to be coupled to the hydrolysis of one molecule of ATP^{1,2}. The distance the actin filament slides relative to the myosin filament in one crossbridge cycle has been estimated as 12 nm by step-length perturbation studies on single fibres from frog muscle^{3,4}. The 'mechanical' power stroke of the attached crossbridge can therefore be defined as 12-nm shortening with a force profile like that shown by the quick recovery of force following a length perturbation. According to this definition, power strokes cannot be repeated faster than the overall ATPase rate. Here, however, we show that the power stroke can be regenerated much faster than expected from the ATPase rate. This contradiction can be resolved if, in the shortening muscle, the free energy of ATP hydrolysis is used in several actin-myosin interactions consisting of elementary power strokes each of 5-10 nm.

Experiments were performed on intact fibres dissected from frog skeletal muscle, a preparation that allows the synchronization of crossbridge action by means of step perturbations in length controlled at sarcomere level³⁻⁷. Figure 1 shows tension transients elicited by single- and double-step changes of sarcomere length imposed on a tetanized fibre. Simultaneously with a step release of ~ 5 nm (Fig. 1A), tetanic tension drops close to zero. This is followed by a quick recovery of tension which is complete within about 2 ms. According to an analysis by Huxley and co-workers^{3,4,8}, the change in force simultaneous with the step (phase 1 of the transient) is mainly due to the elasticity of the crossbridges and the quick recovery (phase 2) is the energy-releasing stroke. With a 5-nm release, the tension attained at the end of phase 2 (T_2) is about 80% of the original tension. During the next 13 ms, tension changes less than 2%. The level of tension attained at the end of quick recovery following a second (test) step of about 2 nm, applied at different times after the first (conditioning) step of 5 nm, is very time-dependent. Only a small part of the tension drop is quickly recovered for a test step imposed 2 ms after the conditioning step. The T_2 level is practically superposable on that attained with a single step of the size corresponding to the sum of test and conditioning steps (Fig. 1B). But T_2 levels increase rapidly (Fig. 1C) with test steps imposed during the next 13 ms. Throughout this whole 15-ms period, the number of the attached crossbridges, estimated by the tension drop occurring simultaneously with the test step (stiffness), changes very little: at 2 ms stiffness is $\sim 90\%$ of the value before the step, whereas at 15 ms it is $\sim 85\%$.

These results can be summarized by plotting the relation of T_2 against the size of the step applied at a given time after the conditioning step. In Fig. 2A, test T_2 curves for three different times are compared with the control T_2 curve (single steps). In the test T_2 curve at 2 ms after the conditioning step, the usual downward concavity³ has disappeared and the intercept on the length axis (x) is reduced from about 11 nm to less than 7 nm. Both the concavity of the T_2 curve and its intercept on the length axis increase with the time elapsed after the conditioning step. By 15 ms the test T_2 curve reattains roughly the same shape as the control curve.

The implications of these results with respect to the regeneration of the crossbridge power stroke is illustrated in Fig. 2B, in which the test T_2 curves of Fig. 2A are shifted to the left by the amount of the conditioning step. The test T_2 curve at 2 ms lies very close to the control T_2 curve, because a step at this time is followed by quick recovery to a tension value that is very similar to that attained at the end of recovery from a single step equal to the sum of the conditioning and the test steps. This behaviour is to be expected if we assume³ that the dominant process, occurring during the 2 ms elapsed from the conditioning step of 5 nm, is a redistribution between different force-

generating states of the attached crossbridges without substantial detachment and reattachment. A release at this time elicits only the portion of the power stroke not consumed by the first step. But within 10–15 ms most of the effect of the conditioning step disappears, whereas stiffness changes very little, which suggests that crossbridges detach and rapidly reattach further along the actin filament and generate another power stroke.

An estimate of the time course of the recovery of T_2 curve can be obtained by plotting the intercept of T_2 curve on the length axis against the time. In this way the recovery of the T_2

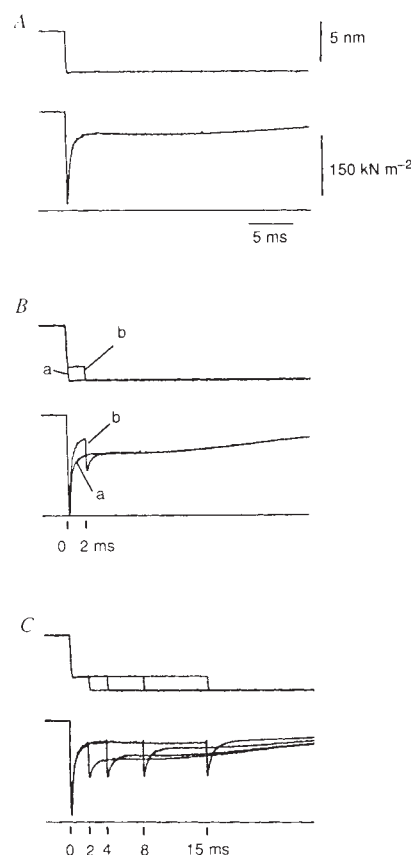


FIG. 1 A, Tension response (middle trace) to a step reduction in length of about 5 nm per half-sarcomere (upper trace) applied to a muscle fibre at the plateau of an isometric tetanus. The lower trace indicates resting tension. B, Superposed records of tension responses (a) to a step release of about 7 nm and (b) to a step of about 2 nm delivered 2 ms after a conditioning step of 5 nm. C, Superposed records of tension responses to test steps of about 2 nm delivered 2, 4, 8 and 15 ms after the conditioning step of 5 nm; same fibre in A, B and C.

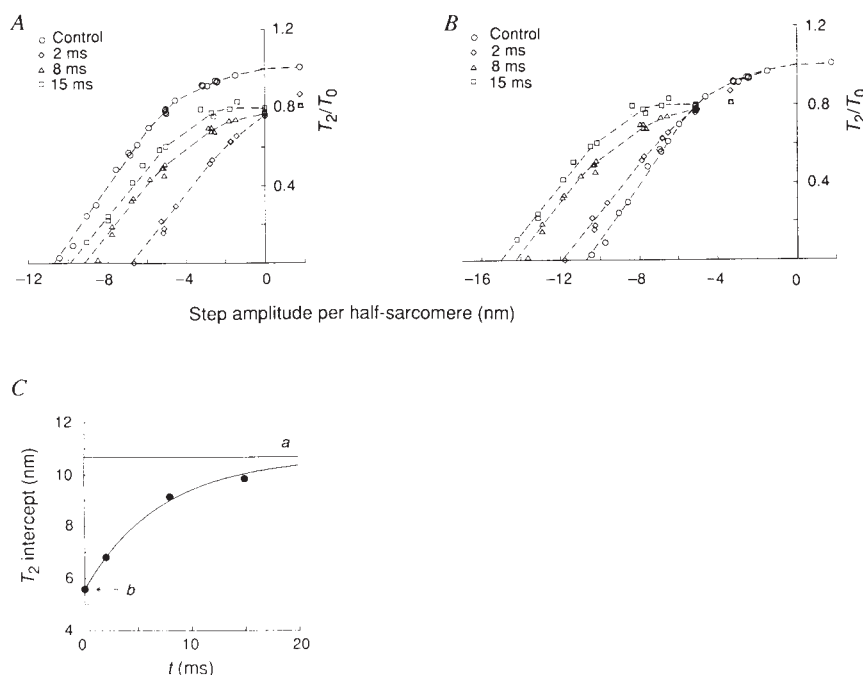
METHODS. Experiments were performed on intact fibres from the lateral head of the tibialis anterior muscle of *Rana esculenta*. In this preparation the striations remain well ordered during contraction. The fibres were mounted on the experimental trough between a loudspeaker motor^{17,18} and a capacitance force transducer with resonant frequency of 40–50 kHz¹⁹. The fibres were stimulated at a sarcomere length of about 2.1 μm with pulse trains of current of suitable amplitude and frequency to produce fused tetani of 0.5–1 s duration. Temperature in the trough was kept at about 4°C by means of a system consisting in a thermoelectric module stuck to the bottom of the experimental chamber, a thermistor probe and a suitable control circuit. Length change of a short segment (0.8–1.5 mm), selected along the fibre close to the force transducer end, was controlled by means of a striation follower⁵, an optoelectronic device that detects the longitudinal translation of the sarcomeres at two points bounding the selected segment. The loudspeaker motor was servocontrolled using feedback from either the position sensor on the loudspeaker lever (fixed-end mode), or the striation follower (length-clamp mode)^{7,18}. In general, fibre stimulation started with the motor operated in fixed-end mode. The control was shifted to length-clamp mode at a preset time during the rise of tetanic tension. When tension had attained the plateau value, step length changes complete in about 110 μs were imposed on the selected segment.

TABLE 1 The time constant (τ) for the regeneration of the T_2 curve after a conditioning step release

Conditioning step (nm)	τ (ms)	T_2/T_0	S_2/S_0
-2.4	8.5	0.92	0.95
-5.0	6.7	0.81	0.92
-5.1	7.2	0.78	0.90
-6.6	4.6	0.61	0.78

T_2 and S_2 represent tension and stiffness at the end of quick recovery. T_2 was determined by the tangent method^{4,16} and was normalized for the isometric plateau value (T_0). S_2 was determined using the smallest of the series of test steps (≤ 1.5 nm) imposed 2 ms after the conditioning step and was measured by the ratio of the tension change occurring simultaneously with the test step to the corresponding sarcomere length change. S_2 was normalized for the value at the isometric tetanus plateau (S_0). Values from four experiments.

FIG. 2 A, T_2 curves determined either from single step ($\circ$) or from test steps delivered 2 ($\diamond$), 8 ($\triangle$) and 15 ($\square$) ms after a conditioning step of about 5 nm. T_2 is the tension attained after quick recovery normalized to the isometric tetanic tension. The dashed lines are drawn by eye through the experimental values. For circles, triangles and squares straight lines are drawn for releases larger than 6 nm; for diamonds the straight line is drawn for releases larger than 3 nm. The intercepts on the abscissa, determined by extrapolation of the straight lines, were: 10.68 nm ($\circ$), 6.8 nm ($\diamond$), 9.15 nm ($\triangle$) and 9.85 nm ($\square$). B, T_2 curves as in A replotted after shifting the test T_2 points to the left by an amount equal to the size of the conditioning step (5.1 nm). C, A plot of the intercept (x) of the T_2 curves from A against the time (t) elapsed after the conditioning step. The horizontal line ($a=10.7$ nm) indicates the value of the intercept of the control T_2 curve. The point at zero time (b) is obtained by subtracting the size of the conditioning step from a ($b=a-5.1=5.6$ nm). The continuous line is fitted to the data according to the equation $x=a-(a-b)\times e^{(-t/\tau)}$. The estimated τ is 7.2 ms.



curve can be approximated by an exponential process with a time constant of about 7 ms (Fig. 2C).

The idea that, after a shortening step, crossbridges detach and rapidly reattach has already been proposed⁹ to explain the finding that the initial drop in 145 Å X-ray reflection (sensitive to the axial repeat of the myosin head), elicited by a step release, is recovered with a time constant of about 6 ms. They also showed that there was a correlation between this time course and the mechanical response to a second step similar to that shown in Fig. 1.

The rate of regeneration of the power stroke increases with the size of the conditioning release, but the fraction of crossbridges undergoing this process decreases because the relative stiffness decreases soon after the conditioning step (Table 1). This can be explained by a fast detachment of crossbridges beyond a critical degree of shortening. The results in Table 1 and in Figs 1 and 2 indicate that: (1) the capacity of crossbridges to do internal work and make the thin filament slide is regenerated at a rate of $\sim 140\text{ s}^{-1}$; (2) the fraction of crossbridges involved in this process is reduced with increase in the size of

the shortening step. With a step release of 5 nm $\sim 90\%$ of crossbridges attached before the step undergoes the quick tension recovery and the subsequent process of regeneration of the power stroke. If the myosin heads attached at the plateau of an isometric tetanus represent a large fraction of the total number of myosin heads¹⁰⁻¹², it is unlikely that the regeneration process is due to new crossbridges attaching and beginning the power stroke after the step. Recent combined mechanical and time-resolved X-ray experiments (M. Irving, V. L., G.P. and M. A. Ferenczi, manuscript submitted), showing a large decrease in 145 Å meridional reflection during phase 2 after a step release, give strong support to the view that a large fraction of myosin crossbridges is attached and participates in the power stroke in an isometric tetanus.

Is there some limit to the number of power strokes interposed with rapid detachment-attachment? This was investigated by measuring tension and stiffness in response to a succession of step releases (staircase shortening) imposed on a contracting fibre. With identical staircase steps of 4–5 nm per half-sarcomere, an apparent steady state response is attained with the third step (Fig. 3). Average steady-state values for tension and stiffness from three experiments, in which staircase steps were spaced at

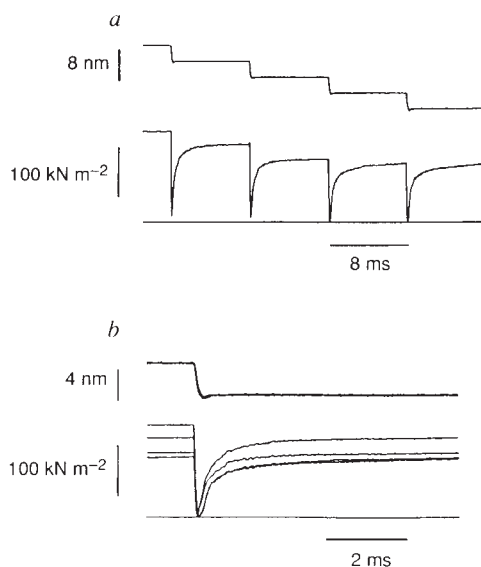


FIG. 3 A, Tension response (middle trace) to a sequence of four step changes of 4.15 nm per half-sarcomere (upper trace) imposed at the plateau of an isometric tetanus. Interval between steps, 8 ms. The lower trace indicates resting tension. B, Superimposition of the steps of record A. Tension (middle traces) and segment length change (upper traces). The tension before the step reduces progressively until a steady value is attained with the third step. In Table 2 are shown data averaged from three fibres in which the same protocol was used. During the 4.43-nm step the force drops from $0.63 T_0$ to approximately zero. Assuming this force decrease occurs linearly, the energy (E_{cb}) released as work per crossbridge is given by

$$E_{cb} = \int F dl = \frac{1}{2}(0.63/0.58)F_0 \times 4.43 \text{ nm}$$

where 0.58 is the fraction of myosin heads attached; F_0 (the isometric force per myosin head) is given by: $T_0/(N \times H) = 1.37$ pN, where T_0 (which equals 206 kN m^{-2} ; see Table 2) is the isometric force per cross-sectional area, N ($500 \times 10^{12} \text{ m}^{-2}$) is the density of myosin filaments and H (300) is the number of myosin heads per myofilament in a half-sarcomere²⁰. E_{cb} is therefore $3.30 \times 10^{-21} \text{ J}$.

TABLE 2 Means ($\pm$ s.e.m.) of tension (T) and stiffness (S) for the first and the fourth of a sequence of identical steps

	T_1/T_0	S_1/S_0	T_2/T_0	S_2/S_0
Step 1	1	1	0.82 ($\pm$ 0.02)	0.89 ($\pm$ 0.02)
Step 4	0.63 ($\pm$ 0.01)	0.72 ($\pm$ 0.01)	0.55 ($\pm$ 0.02)	0.73 ($\pm$ 0.01)

T_1 and S_1 are the values just before the step. T_2 and S_2 are the values at the end of quick recovery following the step. Data from three fibres. The staircase shortening, imposed at the plateau of the isometric tetanus, was made by steps of 4.43 ($\pm$ 0.30) nm per half-sarcomere and intervals of 8 ms. Isometric tetanic force was 206 ($\pm$ 11) kN m $^{-2}$. Temperature 4.2 ($\pm$ 0.2) $^{\circ}$ C. T_2 was determined by means of the tangent method^{4,16}. Tension and stiffness were made relative to the corresponding isometric tetanic values.

8-ms intervals, are reported in Table 2. Assuming that: (1) at the isometric tetanus plateau, 80% of the myosin heads are attached^{10,11}, and (2) the rate of repriming at the steady state of the stair is the same as after a single step of the same size (133 s $^{-1}$ for a step of 4.43 nm, as estimated by fitting a polynomial to the data of Table 1), the correct stroking rate per myosin head will be 133 s $^{-1}$ (0.8×0.73) = 77 s $^{-1}$. The steady-state rate of ATP splitting per myosin head is estimated to be 6 s $^{-1}$ at most^{1,13}. If the maximum ATPase rate during steady shortening is a good estimate of the ATPase rate with our shortening protocol, the stroking rate in our experiments is more than 10-fold higher than ATPase rate. This is consistent with energetic considerations. In the steady state the work produced for each cross-bridge interaction in the experiments of Fig. 3 and Table 2 ($\sim 3 \times 10^{-21}$ J according to the calculation in legend of Fig. 3) is more than 10 times smaller than the energy made available from ATP hydrolysis ($\sim 40 \times 10^{-21}$ J if the efficiency of energy transduction is 50% or more).

During shortening, the free energy provided by ATP hydrolysis is released as work in multiple crossbridge interactions, as if the myosin molecule could retain the potential for doing work even after detaching from actin. At the same time the conventional hypothesis of the myosin head as the motor that makes the actin filament slide by means of an overall axial movement of the order of 10 nm finds strong experimental evidence not only in the mechanical properties³, but also in the recent submillisecond measurements of the meridional 145 Å X-ray reflection accompanying the quick tension recovery following a step release (M. Irving, V.L., G. Piazzesi and M. A. Ferenczi, manuscript submitted). All these results are consistent with elementary power strokes of 5–10 nm, rather than with a power stroke of 60 nm¹⁴, with the possibility of many power strokes per ATP hydrolysed¹⁵. □

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Assembly and function of the two ABC transporter proteins encoded in the human major histocompatibility complex

Adrian Kelly*, Stephen H. Powis*, Lesley-Anne Kerr*, Ian Mockridge*, Timothy Elliott†, Judy Bastin†, Barbara Uchanska-Ziegler‡, Andreas Ziegler‡, John Trowsdale* & Alain Townsend†

* Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK

† Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

‡ Institut für Experimentelle Onkologie Und Transplantationsmedizin, Universitätsklinikum Rudolf Virchow, Freie Universität Berlin, Spandauer Damm 130, DW-1000 Berlin 19, Germany

PRESENTATION of cytoplasmic antigens to class I-restricted cytotoxic T cells implied the existence of a specialized peptide transporter^{1–3} (reviewed in ref. 4). For most class I heavy chains, association with peptides of the appropriate length is required for stable assembly with β_2 -microglobulin^{5–11}. Mutant cells RMA-S (ref. 12) and .174/T2 (refs 13, 14) neither assemble stable class I molecules nor present intracellular antigens, and we have suggested that they have lost a function required for the transport of short peptides from the cytosol to the endoplasmic reticulum^{5–7}. The genetic defect in .174 has been localized to a large deletion in the class II region of the major histocompatibility complex^{6,15,16}, within which two genes (*RING4* and *RING11*) have been identified that code for 'ABC' (ATP-binding cassette) transporters^{15,17–21}. We report here that the protein products of these two genes assemble to form a complex. Defects in either protein result in the formation of unstable class I molecules and loss of presentation of intracellular antigens. The molecular defect in a new mutant, BM36.1, is shown to be in the ATP-binding domain of the *RING11*/PSF2 protein. This is in contrast to the mutant .134 (ref. 15), which lacks the *RING4*/PSF1 protein.

As the deletion in .174 encompassed many genes, we have looked for single, reversible defects in the expression of the two major histocompatibility complex-(MHC)-encoded ABC transporters in other characterized mutant cells. We will refer to these genes as *RING4* and *RING11* (refs 17, 20). To investigate the expression of these genes, we raised an antiserum (AK1.6) against a peptide representing the C-terminal 15 amino acids of the *RING4* protein sequence¹⁷. Immunoprecipitation from extracts of the normal Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line LBL721 revealed two major bands that were absent in extracts from the mutant .174 (Fig. 1a, lanes 1 and 2). As both HLA haplotypes of .174 cells are affected by large deletions that include both of the transporter genes^{15,16}, this result suggested that the two proteins may be encoded by these two loci. The bands migrated with apparent relative molecular masses (M_r) of $\sim 77,000$ (77K; upper) and $\sim 71,000$ (71K; lower) (Fig. 1a, lanes 1 and 2).

The ~ 77 K band from extracts of LBL721 resolved into two species of slightly different size (~ 1.5 K), when separated on 10% polyacrylamide gels. The *RING11* gene has two alleles which should encode proteins that differ in length by 17 amino acids²⁰. These were referred to as 'A' for the shorter and 'B' for the longer form. This difference in length would be sufficient to give rise to the slight difference in migration seen in the two upper bands isolated from LBL 721. We therefore compared a set of ten cell lines with known *RING11* genotypes.

Figure 1b shows that all the cell lines tested gave rise to a band of ~ 71 K. But each cell line had one of three patterns of the upper band(s); A (lanes 2,8), B (lanes 7,10), or A plus B

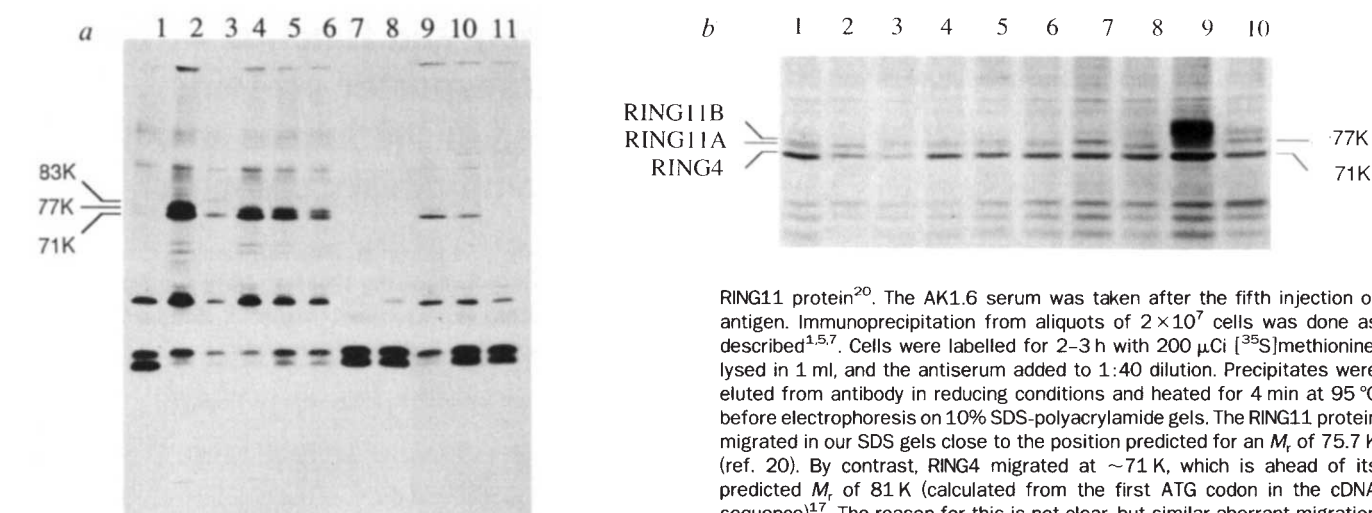


FIG. 1 Detection of the RING4/11 protein complex with the AK1.6 serum to the RING4 polypeptide. *a*, RING4 immunoprecipitates were prepared from the following cell lines: lane 1, mutant .174; lane 2, wild-type cell 721; lane 3, BM36.1; lane 4, BM28.7; lanes 5 and 6, independent populations of BM36.1 cells transfected with a RING11A cDNA; lane 7, T2 cells; lane 8, T2 cells transfected with a RING11A cDNA. In a different experiment, lane 9, wild-type 721; lane 10, T2 cells transfected with a RING4 cDNA; lane 11, T2 cells. The ~71K, ~77K and ~83K bands are marked. *b*, Immunoprecipitates from a series of cell lines genotyped for their *RING11* alleles (in brackets): lane 1, LBL721 (A, B); lane 2, BM28.7 (A, -); lane 3, C1R (A+/-, B; see text); lane 4, PRIESS (A, B); lane 5, STEINLIN (A, B); lane 6, HOM-2 (A, B); lane 7, BASILIO (B, B); lane 8, COX (A, A); lane 9, P.H. (A, B); lane 10, P.W. (B, B). METHODS. The antiserum against RING4 (AK1.6) was raised by a standard procedure. A Sandy half-lop rabbit was immunized with 0.5 mg of peptide GCYWAMVQAPADAPE glutaraldehyde-crosslinked to keyhole limpet haemocyanin (KLH). This sequence is not related to the C terminus of the

RING11 protein²⁰. The AK1.6 serum was taken after the fifth injection of antigen. Immunoprecipitation from aliquots of 2×10^7 cells was done as described^{1,5,7}. Cells were labelled for 2–3 h with 200 μ Ci [³⁵S]methionine, lysed in 1 ml, and the antiserum added to 1:40 dilution. Precipitates were eluted from antibody in reducing conditions and heated for 4 min at 95 °C before electrophoresis on 10% SDS-polyacrylamide gels. The RING11 protein migrated in our SDS gels close to the position predicted for an M_r of 75.7 K (ref. 20). By contrast, RING4 migrated at ~71 K, which is ahead of its predicted M_r of 81 K (calculated from the first ATG codon in the cDNA sequence)¹⁷. The reason for this is not clear, but similar aberrant migration in SDS gels of related proteins in the ABC family does occur²⁴. Finally, we have sometimes seen a protein of ~28 K co-precipitating with transporter proteins from extracts of 721, but not .174. Such a band is visible in *a*, lane 2. We do not yet know the identity of this protein. However, it is in the size range expected for a proteasome subunit²⁵. RING11 genotyping has been described²⁰. Origins of BM36.1 and BM28.7: the BM28.7 cell (HLA-A1, B35/BW6, Cw4) is a hemizygous irradiation mutant of the wild-type EBV-transformed lymphoblastoid cell line BJAB-B95.8.6 (refs 26, 27). BM36.1 was derived from BM28.7 after fractionated irradiations (5 Gy and 2 Gy) and selection for BW6 loss with a monoclonal antibody (SFR8-B6; ref. 28) and complement followed by cloning^{27,29}. Transfection of BM36.1: a sequenced RING11A clone²⁰ was subcloned into the episomal expression plasmid pREP9 (Invitrogen), electroporated into BM36.1 cells and then transfected cells were selected in neomycin. Two independent polyclonal drug-resistant cultures were analysed in these experiments as all the cells in the transfected cultures were found by indirect immunofluorescence to re-express HLA-A1 and B35 at the cell surface.

(lanes 1,4,5,6,9). In nine cases the migration patterns of these bands matched those of the *RING11* genotypes, as determined by sequencing of amplified genomic DNA²⁰. The highly mutated cell line C1R (ref. 22) appeared to be homozygous B by sequence analysis, however, whereas the immunoprecipitates contained band B as well as a weak band A (lane 3). We are investigating this single discrepancy.

The data from the nine cell lines suggested strongly that the

upper band(s) detected in LBL721 by the antiserum against RING4 was encoded by the two alleles of the *RING11* gene, and was co-precipitated with the RING4 protein (lower band). The identification of the lower (~71K) band as the product of the *RING4* gene was confirmed by immunoprecipitation from T2 cells (a derivative of .174 (ref. 14)) transfected with the *RING4* gene (Fig. 1*a*, lane 10). Finally, cross-reaction of the anti-RING4 serum with the RING11 protein was ruled out

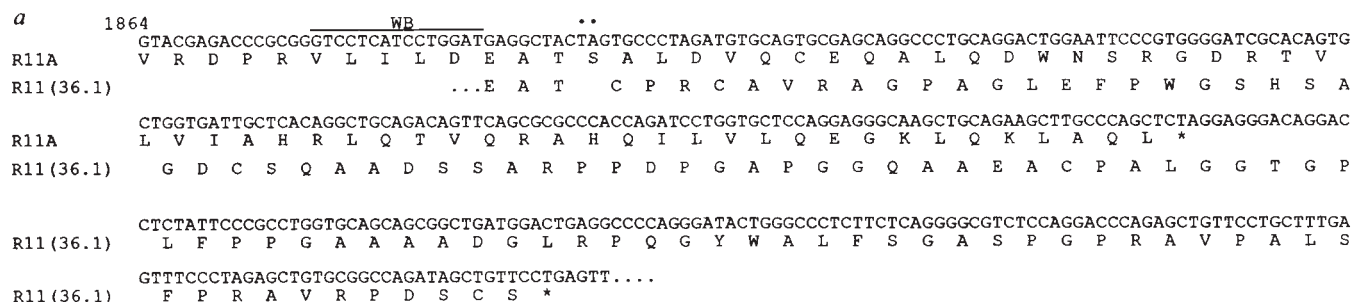
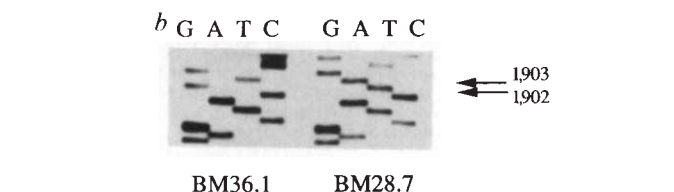


FIG. 2 Sequence of the mutation in the RING11 gene from BM36.1. DNA sequencing: Single-strand cDNA was made from poly(A)⁺ purified BM36.1 RNA using reverse transcriptase and an oligonucleotide primer specific to the noncoding 3' region of *RING11* according to the manufacturer's (Boehringer) protocol. Two further *RING11*-specific oligonucleotides were then used to amplify the entire *RING11* cDNA by polymerase chain reaction (PCR). Portions of the PCR product were re-amplified using RING11-specific biotinylated oligonucleotides and subjected to solid-phase DNA sequencing as described²⁰. The deletion of the two base pairs at nucleotides 1,902 and 1,903 (ref. 20), affecting codons 634 and 635, shown in *a*, was confirmed by solid-phase sequencing of BM36.1 and BM28.7 genomic DNA, shown in



b. The single-letter amino-acid code is used, and the Walker B nucleotide-binding motif is shown²⁰.

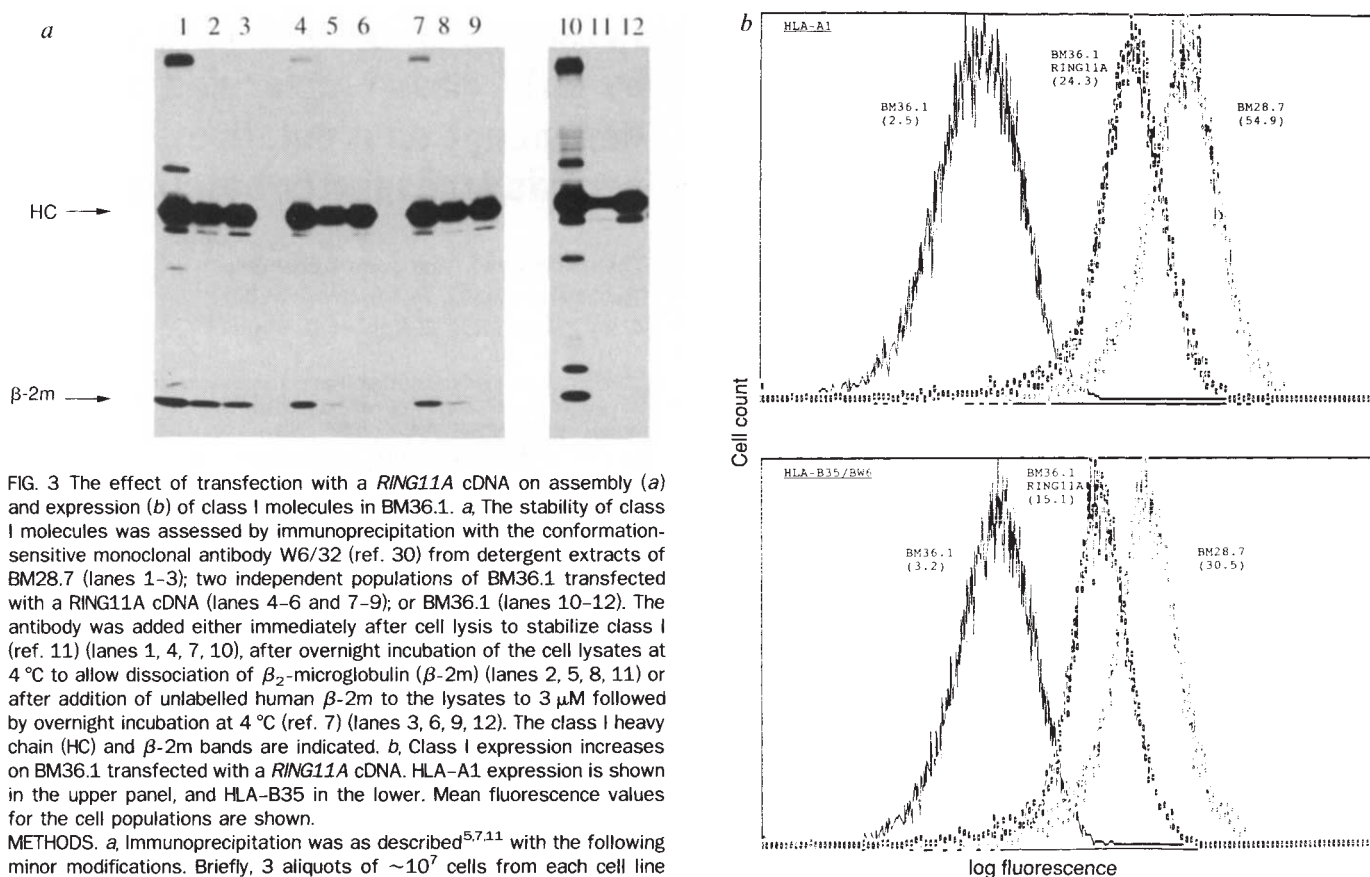


FIG. 3 The effect of transfection with a *RING11A* cDNA on assembly (a) and expression (b) of class I molecules in BM36.1. a, The stability of class I molecules was assessed by immunoprecipitation with the conformation-sensitive monoclonal antibody W6/32 (ref. 30) from detergent extracts of BM28.7 (lanes 1-3); two independent populations of BM36.1 transfected with a *RING11A* cDNA (lanes 4-6 and 7-9); or BM36.1 (lanes 10-12). The antibody was added either immediately after cell lysis to stabilize class I (ref. 11) (lanes 1, 4, 7, 10), after overnight incubation of the cell lysates at 4 °C to allow dissociation of β -2-microglobulin (β -2m) (lanes 2, 5, 8, 11) or after addition of unlabelled human β -2m to the lysates to 3 μ M followed by overnight incubation at 4 °C (ref. 7) (lanes 3, 6, 9, 12). The class I heavy chain (HC) and β -2m bands are indicated. b, Class I expression increases on BM36.1 transfected with a *RING11A* cDNA. HLA-A1 expression is shown in the upper panel, and HLA-B35 in the lower. Mean fluorescence values for the cell populations are shown.

METHODS. a, Immunoprecipitation was as described^{5,7,11} with the following minor modifications. Briefly, 3 aliquots of $\sim 10^7$ cells from each cell line were internally labelled with [³⁵S]methionine, lysed in 1 ml, and the insoluble material removed. Immediately after lysis, purified W6/32 antibody was added to one aliquot to 15 μ g ml⁻¹ (10^{-7} M), human β -2m (Sigma) to a second aliquot to 36 μ g ml⁻¹ (3×10^{-6} M), and to the third aliquot, 25 μ l phosphate-buffered saline as a control. The lysates were incubated at 4 °C overnight and without any preclearing, W6/32 antibody was added to the second and third aliquots to 15 μ g ml⁻¹ for 90 min. The immunoprecipitates were then collected with protein A-Sepharose beads, eluted, reduced and

separated on 12% SDS-polyacrylamide gels. b, Class I expression was measured by indirect immunofluorescence as described¹. The first layer antibodies were 142.2, specific for HLA-A1 (ref. 31) (purified at 5 μ g ml⁻¹), and 4D12 specific for members of the HLA-B5 cross-reactive group³² (as neat culture supernatant). The second layer antibody was fluorescein-labelled, affinity-purified, goat anti-mouse immunoglobulin (Sigma F0257) at 1:40 dilution.

because neither band precipitated from T2 cells transfected with a *RING11A* gene (Fig. 1a, lanes 7,8).

Further experiments with a pair of mutant lymphoblastoid cell lines, BM36.1 and BM28.7, confirmed the identity of the RING11 band and provided evidence for a function of the RING11 protein. BM36.1 was derived from the hemizygous cell BM28.7 (see legend to Fig. 1) and has a phenotype similar to RMA-S (refs 5, 7, 12). The class I molecules synthesized and assembled in BM36.1 are unstable *in vitro* (Fig. 3a), are present at low levels at the cell surface (Fig. 3b), and do not present intracellular influenza proteins to HLA-A1-restricted cytotoxic T cells (Fig. 4c).

Immunoprecipitation with the anti-RING4 serum from extracts of BM36.1 revealed that the ~ 77 K RING11 band was missing from this cell line, but was present in the precursor hemizygous cell line BM28.7 in the A form (Fig. 1a, lanes 3 and 4; Fig. 1b, lane 2). But a unique band of higher M_r (~ 83 K, see Fig. 1a) was co-precipitated from extracts of BM36.1. Transfection of BM36.1 with an A allele of *RING11* restored the RING11A band in immunoprecipitates as expected, with associated loss of the protein with higher M_r (Fig. 1a, lanes 5 and 6).

Analysis of genomic DNA by Southern analysis from BM36.1 and BM28.7 revealed no abnormalities in the *RING11* gene, and *RING11A* messenger RNA expression was indistinguishable in the two cell lines (data not shown). But sequence analysis of the RING11 complementary DNA from BM36.1 revealed a

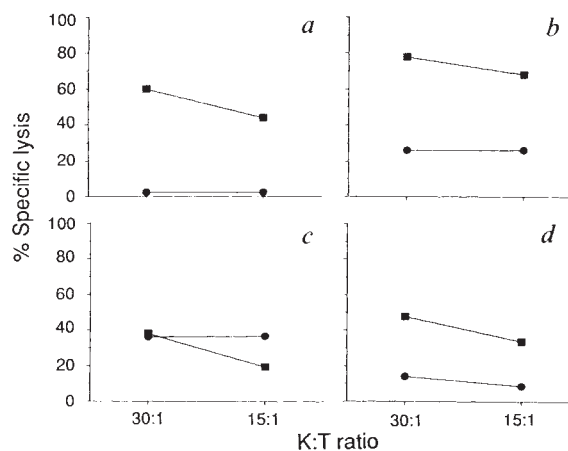


FIG. 4 Presentation of influenza antigens in BM36.1 transfected with a *RING11A* cDNA. An HLA-A1-restricted cytotoxic T-lymphocyte (CTL) line was tested for recognition of influenza-infected (squares) or uninfected (circles) cell lines: a, autologous EBV-transformed lymphoblastoid cell line; b, BM36.1 transfected with a *RING11A* cDNA; c, BM36.1; and d, BM28.7.

METHODS. Fresh peripheral blood lymphocytes obtained from a donor (HLA-A1, A3, B8, B60) were stimulated *in vitro* with influenza AX31 as described³³. After 7 days the cells were restimulated with an HLA-A1-restricted peptide derived from the influenza A nucleoprotein (amino acids 89-101; J.B., unpublished results) and after a further 7 days the CTL were tested in a ⁵¹Cr release assay for recognition of influenza AX31-infected target cells³³. Spontaneous ⁵¹Cr release in the absence of CTL ranged between 22%-35%.

deletion of two base pairs at codons 634–635, which lies immediately 3' to the Walker B nucleotide-binding motif in the RING11A sequence²⁰ (Fig. 2). The consequent change in reading frame resulted in replacement of the C-terminal 52 amino acids of the RING11A ATP-binding domain with new sequence, and a 51-amino-acid extension to the protein. This was consistent with the increase in M_r of ~6K seen in the unique protein co-precipitated from BM36.1 with the anti-RING4 serum (Fig. 1a, lane 3 and Fig. 2). The loss of the ~83K form of RING11 after transfection with a normal gene (Fig. 1a) suggests that the wild-type protein competed efficiently with the elongated mutant for binding to the RING4 protein.

The mutant HLA phenotype of BM36.1 was reversed by transfection with a RING11A cDNA clone. The proportion of stable intracellular class I molecules in BM36.1 rose (Fig. 3a), and class I expression at the cell surface (Fig. 3b) was restored to ~50% of the level on the hemizygous precursor BM28.7. Finally, presentation of influenza antigens to an HLA-A1-restricted cytotoxic T-cell line was restored (Fig. 4b) to levels comparable to the BM28.7 precursor (Fig. 4d), or the normal HLA-matched lymphoblastoid cell line (Fig. 4a).

Our results show that the RING4 and RING11 proteins form a complex, but do not reveal its stoichiometry. Loss or mutation of either protein results in the loss of a function required for assembly of stable class I molecules and presentation of intracellular antigens. The mutation in the ATP-binding domain of the RING11 protein in BM36.1 suggests that ATP hydrolysis may be required for this function *in vivo*. The reversal of the mutant phenotype by transfection, the stabilization of class I molecules from the mutant cells by peptides *in vitro*^{5,7,8}, and the structural similarities of the RING4 and RING11 proteins to other proteins with transport functions²², all suggest that the RING4/11 complex transports peptides from the cytosol to the endoplasmic reticulum^{1,5}. □

Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer

Thomas Spies*, Vincenzo Cerundolo†, Marco Colonna*‡, Peter Cresswell§, Alain Townsend† & Robert DeMars||

* Division of Tumor Virology, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115, USA

† Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

‡ Istituto Nazionale per la Ricerca sul Cancro, Genova 16132, Italy

§ Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06510, USA

|| Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706, USA

MAJOR histocompatibility complex (MHC) class I molecules present peptides derived from the endogenous protein pool to cytotoxic T lymphocytes, which can thus recognize intracellular antigen^{1–3}. This pathway may depend on a transporter (PSF1) (refs 4–6) to mediate entry of the cytosolic peptides into a pre-Golgi compartment where they bind to class I heavy chains and promote their stable assembly with β_2 -microglobulin^{7–12}. There is, however, only indirect support for this function of PSF1 (ref. 6). Here we show that PSF1 is necessary for the efficient assembly of class I molecules and enables them to present a peptide epitope derived from endogenously synthesized viral antigen. Immunochemical and genetic data demonstrate that the PSF1 polypeptide is associated with a complementary transporter chain, which is polymorphic and is encoded by the *PSF2* gene¹³, which is closely linked to *PSF1*.

Impaired surface expression of class I molecules is associated with a transcriptionally inactive single *PSF1* gene in the MHC-hemizygous human B-lymphoblastoid cell line (LCL) mutant 721.134 (refs 4, 14). Normal abundance of surface HLA-A2 and HLA-B5 can be restored in these cells by gene transfer of *PSF1* (ref. 6). This supports the proposed role of *PSF1* in supplying nascent class I chains with cytosolic peptides, but the evidence is indirect. We therefore compared the ability to present endogenous viral antigen of mutant .134 cells and a derivative transfectant cell line (2C2) with reconstituted expression of *PSF1*.

In cytotoxicity assays, we examined presentation of the M58–68 peptide epitope of influenza virus matrix protein¹⁵ to a specific HLA-A2-restricted T-cell line after infection of target cells with recombinant vaccinia virus directing synthesis of matrix protein. In several experiments, 2C2 cells were specifically lysed about as efficiently as the parental LCL wild-type 721 cells (Fig. 1b, d). HLA-A2 expression in 2C2 cells was therefore functionally normal. By contrast, .134 cells were reproducibly resistant to lysis, like LCL mutant 721.174 cells¹⁶, although low cytotoxicity levels above background were recorded with .134 cells but not with .174 cells (Fig. 1a, c). All target cell lines were efficiently lysed in the presence of extracellular M58–68 synthetic peptide (Fig. 1a–d). Thus, *PSF1* was required for processing of the intracellular antigen. As peptide M57–68 without a signal sequence fails to be translocated from the cytosol into an exocytic compartment in mutant .174-derived T2 cells¹⁷, these results support a physiological function of *PSF1* in peptide transport.

Association with peptides is necessary for stable assembly of class I molecules^{10,12,16,18,19}, which was used as a direct measure of the apparent deficiency of peptides in .134 cells⁶. In detergent lysates of .134 cells, the amounts of HLA-A2 heavy chain- β_2 -

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microglobulin (β_2m) complex detected with the BB7.2 monoclonal antibody²⁰ were much lower than in samples of 721 cells (Fig. 2). Addition of β_2m increased the quantity of HLA-A2 heterodimers, indicating that unstable complexes were present. Similarly, peptide K62 (a derivative of peptide M58-68) enhanced pairing of HLA-A2 heavy chains with β_2m and/or stabilized low-affinity HLA-A2 heterodimers without peptides (Fig. 2). Thus, assembly of HLA-A2 molecules was impaired because of lack of suitable peptides in .134 cells, in agreement with the phenotypes of the mutant .174 and RMA-S cell lines^{16,18,19}. In contrast, efficient assembly of HLA-A2 molecules was restored in 2C2 cells and in an independent transfectant isolate, 2C5 cells (Fig. 2). These results therefore indicate a role of PSF1 in supplying naturally processed peptides promoting stable assembly of class I molecules.

Genetic evidence suggests that PSF1 may not function independently⁶. To investigate an association of PSF1 with other polypeptides, we carried out immunoprecipitations with a specific anti-peptide antiserum. Several bands seen in material from 721 and LCL 721.45 cells¹⁴ were missing in lanes carrying samples from mutant .134 and .174 cells (Fig. 3; similar results were obtained from .134 cells with the AK1.6 antiserum²¹). A major band corresponding to a relative molecular mass of ~68,000 (68K) was restored in 2C2 and 2C5 cell samples and in a transfectant derivative of .174 cells (4B6) selected for expression of PSF1 messenger RNA (Fig. 3)⁶. This band there-

fore corresponds to PSF1. Two additional bands of ~76K and 74K from 721 cells segregated with the alternate single MHC haplotypes of the hemizygous .45 and LCL 721.112 cells, respectively^{14,22}. The 76K band was restored in immunoprecipitates from 2C2 and 2C5 cells, but not from 4B6 cells (Fig. 3). Thus, there are two allelic variants of a protein that is associated with PSF1 and maps to the homozygous deletion in mutant .174 cells. This is supported by the absence of both the 76K and 74K bands from LCL mutant 5.2.4 cells (Fig. 3), in which a large deletion similar to that in .174 cells includes *PSF1* among several other genes^{4,23}.

These findings correlate with the location of the *PSF2* transporter gene¹³ near *PSF1* and the existence of a *PSF2* amino-acid-sequence length polymorphism. The C-terminal 17 amino acids of the full-length *PSF2*(long) sequence are absent in *PSF2*(short) because of substitution of Gln 687 by a stop codon (M.C. and T.S., unpublished results). By oligonucleotide-typing of genomic DNA, the haplotypic distribution of the *PSF2*(l) and *PSF2*(s) alleles precisely agreed with the immunochemical data (Fig. 4). Taken together, these results therefore establish a physical association of the *PSF1* and *PSF2* polypeptide chains, indicating that these function as complementary subunits. This is supported by the inadequacy of *PSF1* alone to restore normal expression of class I molecules in 4B6 cells and the coregulation of *PSF1* and *PSF2* by interferon- γ (refs 6, 13). Moreover, *PSF1* and *PSF2* each consist of single hydrophobic membrane-anchored and hydrophilic ATP-binding domains¹³; a duplicated arrangement of these two domains is probably universal in all the other members of the multidrug-resistance family of transporters²⁴.

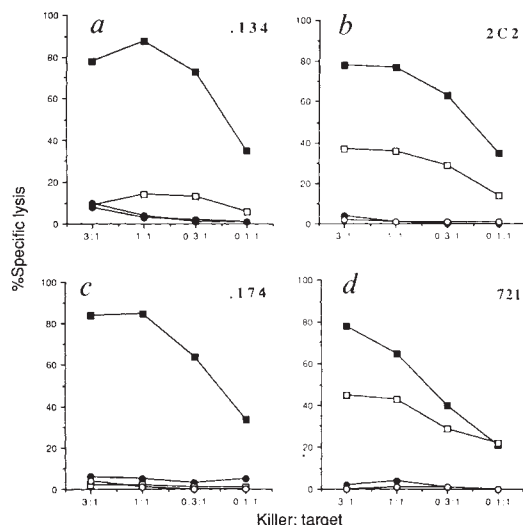


FIG. 1 Gene transfer of PSF1 restores presentation of intracellular antigen by mutant .134 cells. *a*, The JM49 T-cell line specific for the influenza virus matrix protein epitope M58-68 lysed .134 cells exposed to peptide M58-68, but not after infection with recombinant vaccinia virus (M1-vac) directing synthesis of matrix protein. *b*, Presentation of the endogenous antigen was restored in .134-derived 2C2 cells expressing PSF1. *c*, Like .134 cells, mutant .174 cells failed to present the endogenous antigen. *d*, Normal antigen presentation by parental wild-type 721 cells. Target cells were: □, infected with M1-vac; ■, treated with peptide M58-68; ○, infected with the negative control virus LAC-vac; ●, untreated.

METHODS. 2C2 cells were isolated by stable transfection of .134 cells with the cDNA expression construct RSV.5(gpt)-PSF as before⁶. The quantity of HLA-A2 on 2C2 cells was equivalent to that on .45 cells, as measured by binding of the BB7.2 and MA2.1 monoclonal antibodies^{20,25} and flow cytometry (data not shown). JM49 is an influenza A virus-specific HLA-A2-restricted T cell line, which specifically recognizes the matrix 1 epitope 58-68 (ref. 15). The activity of JM49 cells was tested by a standard ⁵¹Cr-release assay with target cells exposed to peptide (5×10^{-5} M) for 1 h or infected with M1-vac (5 plaque-forming units per cell)¹⁶ or LAC-vac (5 PFU per cell) directing expression of bacterial β -galactosidase²⁶. ⁵¹Cr-labelled target cells were plated at 1×10^4 cells per well. The killer to target ratios are indicated. Supernatants were collected after 4 h and the per cent specific lysis was calculated as described¹⁰.

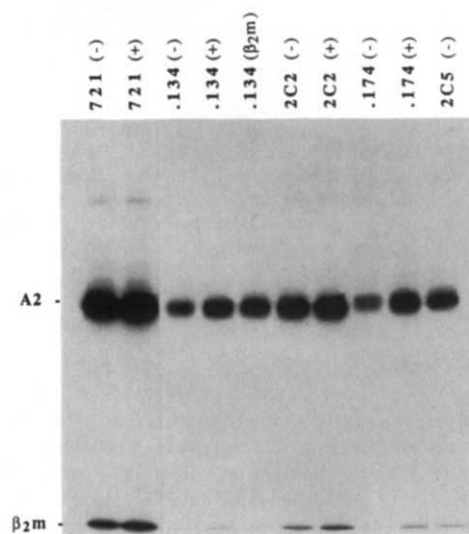
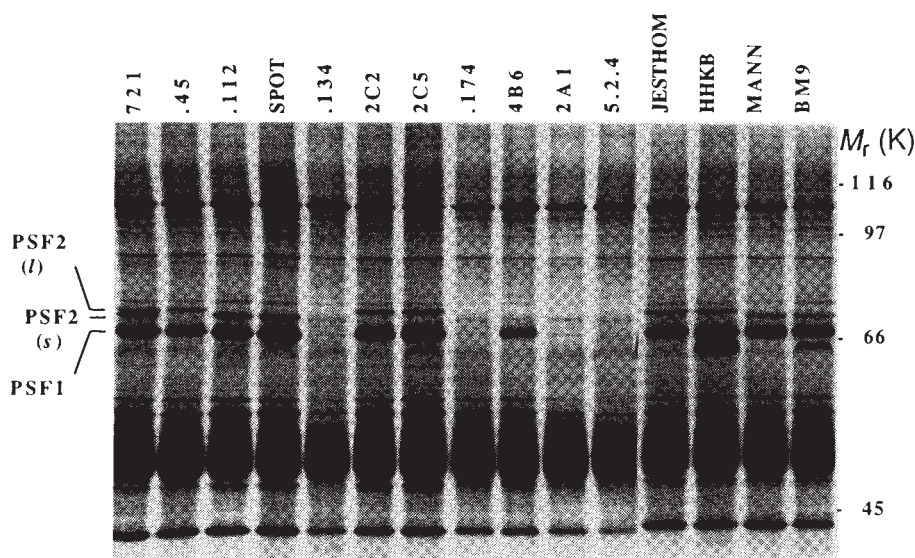


FIG. 2 Gene transfer of PSF1 restores stable assembly of HLA-A2 molecules in mutant .134 cells. The quantity of HLA-A2- β_2m heterodimers detected with the specific heavy chain conformation-dependent BB7.2 monoclonal antibody²⁰ in .134 cells was about as low as in mutant .174 cells¹⁶ and was increased by addition of either β_2m or peptide K62. Transfectant 2C2 and 2C5 cells contained larger amounts of HLA-A2- β_2m heterodimers, independent of exogenous peptide. These levels were lower than in wild-type 721 cells, possibly indicating a dosage effect related to the absence of one MHC haplotype. (-), No peptide added; (+), with peptide K62; A2, HLA-A2 heavy chain.

METHODS. Detergent lysates of samples of 7.5×10^6 cells labelled with [³⁵S]methionine (100 μ Ci) for 1 h were prepared as described¹⁹. Where indicated, peptide K62 (ref. 19) or human β_2m (Sigma) were added to lysates at the final concentrations of 50 μ M and 5 μ M, respectively. After 1 h of incubation on ice, samples were pre-cleared and immunoprecipitated¹⁹ using purified BB7.2 monoclonal antibody (15 μ g ml⁻¹). Immunocomplexes were denatured and analysed by electrophoresis in SDS-polyacrylamide gels (12%). Fixed and stained gels were treated with Amplify (Amersham), dried and exposed to pre-flashed X-ray film.

FIG. 3 Identification of PSF1-PSF2 heterodimers or heteromultimers by immunoprecipitation of PSF1. The 68K band absent from the lanes of mutant .134, .174 and 5.2.4 cells corresponds to PSF1. Expression of PSF1 is restored in .134-derived transfectant 2C2 and 2C5 cells and in .174-derived transfectant 4B6 cells. Bands of 76K and 74K in the lane of 721 cells are dependent on PSF1 and correspond to the PSF2(l) and PSF2(s) alleles, respectively. These segregate with the alternate single MHC haplotypes of either .45 or .112 and SPOT cells (refs 14 and 22; and R.D., unpublished data). 2A1 cells were derived from .174 cells by stable transfection with a PSF2(l) cDNA expression construct (T.S., unpublished). JESTHOM, HHKB, MANN and BM9 are typing cell lines homozygous for PSF2(l) or PSF2(s). Numbers on the right indicate molecular weight standards in thousands.

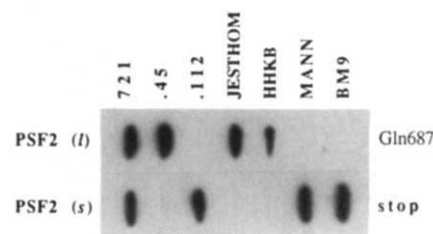
METHODS. The anti-peptide antiserum RING4C was produced by immunizing rabbits²⁷ with a synthetic peptide corresponding to the C-terminal 14 residues of the decoded RING4 cDNA sequence, which is identical to PSF1 (refs 4, 5, 13). For affinity purification, peptide was coupled through the sulphydryl group of the N-terminal cysteine residue to the terminal amino groups of ω -amino butyl agarose (Sigma) activated with the crosslinking reagent SPDP (3-(2-pyridyldithio)-propionic acid N-hydroxysuccinimide ester (Sigma)). Antibodies were eluted with 3.5 M MgCl₂ and were used at 5 μ g per immunoprecipitation. Detergent lysates of 2×10^7 cells



were prepared and precleared for protein analysis¹⁹. Immunocomplexes were isolated with protein A-Sepharose, washed, denatured and electrophoresed in 0.75-mm SDS-polyacrylamide gels (10%). Gels were fixed and subjected to two cycles of silver staining²⁸.

FIG. 4 Typing of genomic DNA for PSF2(l) and PSF2(s). The allelic distribution of PSF2(l) and PSF2(s) corresponds to the presence or absence of the 76K and 74K protein bands shown in Fig. 3. In PSF2(s), glutamine at position 687 of the PSF2(l) sequence¹³ is replaced by a stop codon because of a C $\rightarrow$ T transition at position 2,155 of the nucleotide sequence (M.C. and T.S., unpublished data).

METHODS. The cDNA-derived primers for DNA amplification by the polymerase chain reaction (PCR) were ACAGTCTGGTGATTGCTC (5', nucleotides 2,059-2,077) and CACAGC-TCTAGGGAACTC (3', nucleotides 2,291-2,273) (ref. 13). Genomic DNA samples (0.5 μ g) were subjected to PCR (33 cycles) with Ampli-Taq polymerase (Perkin Elmer Cetus) in the presence of 0.5 μ M primers. Specific amplified fragments were gel-purified, denatured and applied to Hybond membranes (Amersham) using a slot-blot apparatus (Schleicher and Schuell). Membranes were hybridized with ³²P-labelled oligonucleotides (GTCCCT-CCT(G/A)GAGCTGGG) specific for PSF2(l) and PSF2(s).



On the basis of the high steady-state levels of PSF1 detected by direct protein staining (Fig. 3), there are an estimated several hundred thousand transporter molecules in a single cell of the here examined B-lymphoblastoid cell lines. The observed size of PSF2(l) agrees with the value of 77K calculated from the translated cDNA sequence, but the apparent size of PSF1 of 68K does not fit with the predicted 81K (ref. 13). This difference could result from *in vitro* proteolytic cleavage or a post-transla-

tional alteration of PSF1. In conclusion, our results demonstrate the existence and functional integrity of PSF1-PSF2 heterodimers or heteromultimers controlling the endogenous pathway of antigen presentation by class I molecules. Although the 'peptide supply factor' function of PSF1 and PSF2 is evident, their proposed role in peptide transport has still to be directly verified. □

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Ham-2 corrects the class I antigen-processing defect in RMA-S cells

Michelle Attaya*, Stephen Jameson†, Coleen K. Martinez*, Evan Hermel‡, Carla Aldrich§, James Forman§, Kirsten Fischer Lindahl§||¶, Michael J. Bevan† & John J. Monaco*#

* Department of Microbiology and Immunology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0678, USA

† Howard Hughes Medical Institute, Department of Immunology, University of Washington, Seattle, Washington 98195, USA

|| Howard Hughes Medical Institute, Departments of § Microbiology and

¶ Biochemistry, and the ‡ Graduate Immunology Program, University of Texas Southwestern Medical Center, Dallas, Texas, 75235-9050, USA

THE murine major histocompatibility complex (MHC) contains two genes (*Ham-1* and *Ham-2*) that encode members of a superfamily of ATP-dependent transport proteins^{1,2}. These genes are believed to mediate the transport of peptide antigen from the cytoplasm into the lumen of the endoplasmic reticulum for binding by MHC class I molecules. Evidence for such a function has come from the rescue of class I surface expression by a cloned copy of the human homologue of *Ham-1*, *PSF-1*, in a human cell line that is defective in antigen processing³. A mutant murine cell line, RMA-S, has an identical antigen-processing-defective phenotype^{4,5}. Here we show that expression of a cloned copy of the *Ham-2* gene in RMA-S cells results in recovery of the ability to process and present class I-restricted antigens to cytotoxic T lymphocytes, and in partial recovery of class I surface expression. Processing defects for classical (H-2 K and D) and non-classical (Qa1 and HMT) class I molecules are corrected by *Ham-2*. These data indicate that both MHC-linked transporter genes are probably required for class I antigen processing, and that the functional transporter in this pathway may consist of a *Ham-1*/*Ham-2* heterodimer.

The *Ham-2* gene is ~10 kilobases (kb) telomeric to the related *Ham-1* gene in the murine MHC². Both the relative positions and sequence comparisons indicate that *Ham-1* is the murine homologue of the human *PSF-1* (also called Y3 or *RING4*) gene, and that *Ham-2* is the homologue of *PSF-2* (Y1 or *RING11*) (ref. 6). We have previously suggested¹ that defects in either or both of these genes could lead to a phenotype that is defective in antigen processing, as in several human and the RMA-S cell lines^{5,7-9}. These mutants are defective in the assembly of stable MHC class I molecules, presumably because of an inability to provide class I heavy and light (β_2 -microglobulin (β_2m)) chains with appropriate peptides. Reduced numbers of empty (peptide-less) class I heterodimers are transported to the cell surface, where the two chains dissociate and rapidly undergo conformational changes at 37 °C. Such empty class I heterodimers are more stable at reduced temperature (22–26 °C), and accumulate at the cell surface under these conditions^{10,11}. Incubation of the cells in the presence of both an appropriate peptide and β_2 -microglobulin^{12–14} results in the spontaneous assembly of stable class I molecules (with bound peptide), thus restoring their ability to serve as targets for the corresponding antigen-specific cytotoxic T lymphocytes (CTL), and partly restoring cell-surface class I expression.

In one such cell line, class I surface expression is restored by transfection of complementary DNA corresponding to the *PSF-1* (*Ham-1*) gene³. But the same cloned gene does not restore class I expression to the mutant line 721.174, which has deleted both transporter genes⁸, and a cloned *Ham-1* gene does not correct the defect in RMA-S cells (data not shown). The defect in

RMA-S does, however, map to chromosome 17, which includes the *H-2* complex¹⁵. These results strongly suggest that a defect in another gene linked to, but distinct from, *Ham-1* can cause an identical antigen processing-defective phenotype.

Introduction of a full-length *Ham-2* cDNA clone into RMA-S results in transfectants with increased cell-surface class I expression (Fig. 1). We chose one *Ham-2* transfectant, Q11, for further study. Q11 has increased amounts of H-2K^b and H-2D^b molecules at the surface (Fig. 2). Additionally, these class I molecules contain murine β_2m , as demonstrated by reactivity with antibody AF6 (ref. 14) (Fig. 2) and S19.8 (ref. 16, and data not shown). This observation implies that (at least for K^b) the increase in class I surface expression derives from completely assembled molecules reaching the cell surface, not from binding of peptide at the cell surface, in which case the resulting molecules would be expected to contain bovine β_2m (derived from the fetal bovine serum) rather than murine β_2m . Hence,

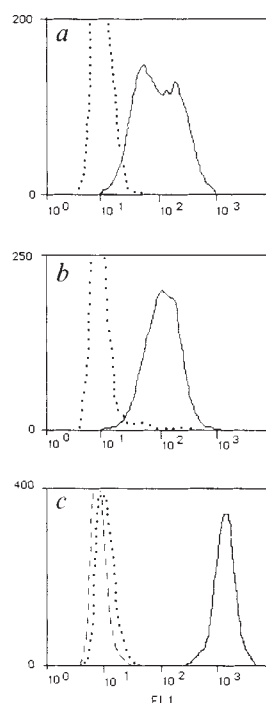


FIG. 1 *Ham-2* transfection of RMA-S results in increased cell-surface expression of class I molecules. Two transfectant clones, Q10 (a), and Q11 (b), were stained with monoclonal antibody 34-1-2S, which recognizes K^b molecules¹⁸, followed by dichlorotriazinylaminofluorescein-conjugated F(ab')₂ fragments of goat anti-mouse IgG (Jackson ImmunoResearch) (solid lines), or with second-stage antibody only (dotted lines), and analysed on a Becton-Dickinson FACSscan with immunofluorescence on a logarithmic scale. c, FACSscan profiles for RMA-S (solid line) and RMA-S (dotted line) stained with 34-1-2S and RMA-S stained with second stage antibody only (dashed line). Graphs show green fluorescence (FL1) on x axis, and cell number on y axis. METHODS. A full-length (2.4 kb) *Ham-2* cDNA clone (M.A. and J.J.M., manuscript in preparation) isolated from a 3T3 Swiss albino fibroblast cDNA library (Clontech) was inserted into the *EcoRI* site of plasmid pCDNA1Neo (In Vitrogen). RMA-S cells (4×10^6) were suspended in 200 μ l RPMI 1640 culture medium (bicarbonate-buffered RPMI 1640 containing 2 mM glutamine, 100 units per ml penicillin, 100 μ g ml⁻¹ streptomycin and 10% fetal calf serum) containing 10 μ g plasmid DNA, and electroporated at 250 V, 500 μ F using a BioRad gene pulser. Electroporated cells were diluted to 14 ml in culture medium, grown for 36 h before addition of antibiotic G418 (Sigma) to 1.1 mg ml⁻¹, and aliquoted into 24-well culture plates. Antibiotic-resistant colonies grew up in 33% of the wells, and all of these showed increased surface expression of class I. Q10 and Q11 are representative of the positive clones. We did not obtain any clones in which class I surface expression returned to the level on RMA cells, even after co-transfection with *Ham-1* and *Ham-2* (data not shown).

* To whom correspondence should be addressed.

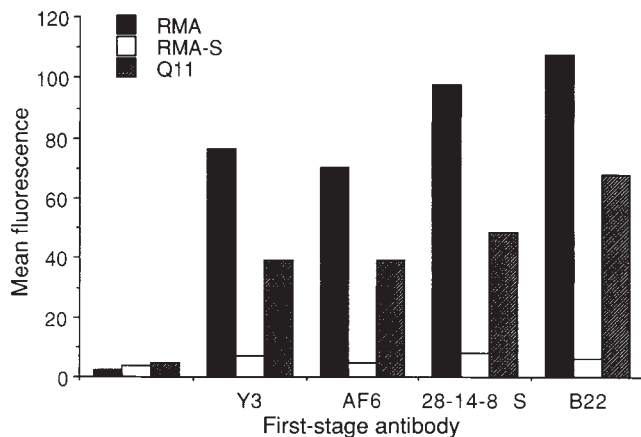


FIG. 2 Cell-surface expression of both K^b and D^b is increased in RMA-S cells transfected with *Ham-2*. RMA, RMA-S and the *Ham-2*-transfected RMA-S clone Q11 were assayed by FACS analysis for expression of K^b with monoclonal antibodies Y3 (ref. 19) and AF6-88.5 (ref. 14) and for expression of D^b with monoclonal antibodies 28-14-8S (ref. 20) and B22-249 (ref. 21). Cells (10^5) were incubated with the antibodies indicated for 45 min at 4 °C, washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel) and analysed. Values shown are mean fluorescence. Similar results were obtained in radioimmunoassays with monoclonal antibodies specific for K^b (B8-24-3; ref. 22) and mouse β_2m^b (S19-8; ref. 16).

the introduced *Ham-2* gene apparently relieves the block in processing of endogenous antigen in RMA-S cells.

Analysis of the ability of Q11 cells to present endogenously processed antigens to class I-restricted CTL supports this conclusion. Both ovalbumin-specific (K^b -restricted), and influenza nucleoprotein-specific (D^b -restricted) CTL lyse Q11 targets essentially as effectively as wild-type RMA cells, whereas mutant RMA-S targets are not recognized (Fig. 3). In addition, CTL specific for the class I alloantigens H-2^b and Qa1^b, and for Mta, a minor histocompatibility antigen (MTF^a) presented by the HMT^a class I molecule¹⁷, are all capable of lysing Q11 and RMA, but not RMA-S targets (Fig. 4). Q11 is not sensitive to nonspecific lysis by H-2^b anti-H-2^d or anti-H-2^{cas3} CTL (not shown). Thus Q11 cells have simultaneously recovered the ability to be recognized by CTL specific for at least five different peptide antigens in the context of at least four different MHC class I molecules.

We conclude that the *Ham-2* gene completely restores the ability of RMA-S to process and present antigen to class I-restricted T cells. The failure to restore wild-type levels of class

I surface expression in these experiments may be due to the presence of secondary genetic defects in RMA-S cells that reduce surface expression of class I molecules by a mechanism other than inhibiting antigen processing. Indeed, during the derivation of RMA-S cells from the wild-type parental line (five rounds of negative selection with anti-class I antibody plus complement), cell lines with intermediate levels of class I surface expression were obtained, suggesting that multiple mutations are responsible for the ultimate phenotype of RMA-S. The surface expression of class I on the Q11 transfectant line resembles that of RMA cells after the first two rounds of negative selection⁴. But we cannot rule out other potential explanations, such as possible sequence defects in, or low-level expression of, the transfected cDNA.

A defect in either *Ham-1* alone (as in mutant 721.134 (ref. 3)) or in *Ham-2* alone (RMA-S, this study) yields cells with indistinguishable phenotype. Furthermore, these mutants are identical in phenotype to a mutant (721.174 (ref. 7)) in which both of the corresponding genes are deleted. The simplest interpretation of these results is that the class I antigen processing

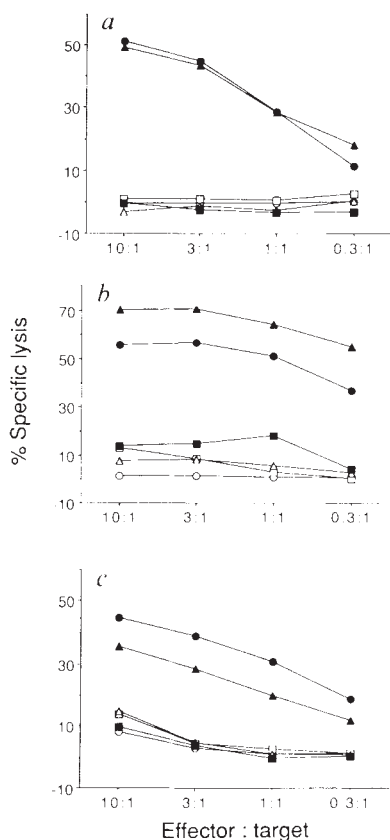
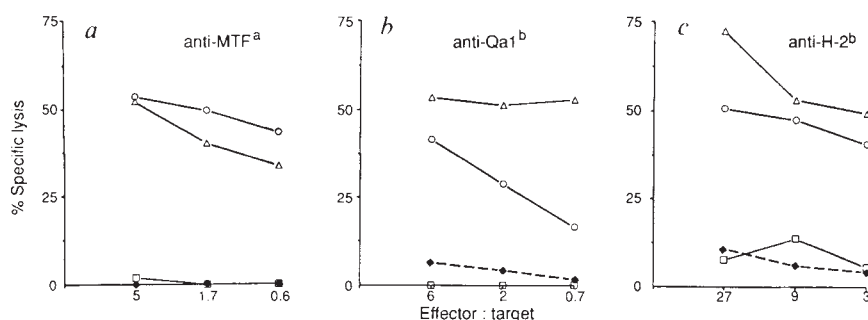


FIG. 3 *Ham-2*-transfected RMA-S cells can present both K^b - and D^b -restricted antigens to class I-restricted CTL. RMA (circles), RMA-S (squares) and the *Ham-2* transfected RMA-S clone Q11 (triangles) were used as targets in a 3-h ⁵¹Cr-release assay at the indicated effector-to-target ratios. **a**, Target cells were loaded²³ in the absence (open symbols) or in the presence (filled symbols) of 3 mg ml⁻¹ ovalbumin (Ova), and tested for lysis by the Ova/ K^b -specific CTL clone GA4. **b**, Untreated targets (open symbols) or targets infected with influenza virus strain A/HK/8/68, expressing nucleoprotein NP1968, (filled symbols), were tested for lysis by a D^b -restricted CTL line specific for NP1968. **c**, Targets were infected with influenza virus strain A/HK/8/68 (open symbols) or strain A/PR/8/34, which expresses NP1934, (filled symbols), and tested for lysis by a D^b -restricted CTL line specific for NP1934.

METHODS. The GA4 CTL clone was maintained on E.G7-Ova cells as described²⁴. Influenza-specific CTL lines were established by priming and restimulating²⁵, and maintained on C57BL/6 spleen cells coated with synthetic peptides²⁶ corresponding to amino acids 366–374 of NP1968 for anti-A/HK/8/68 CTL or of NP1934 for anti-A/PR/8/34 CTL. Target cells were virally infected as described²⁷. As positive controls, all target cells were assayed in parallel with peptides (at 200 nM) ova 257–264 (ref. 28), recognized by GA4, and the influenza NP peptides (already described for the anti-influenza lines). Under these conditions, GA4 lysed all targets to >50% at an effector-to-target ratio of 10:1, and both anti-influenza lines lysed all targets to >50% at a ratio of 1:1.

FIG. 4 *Ham-2*-transfected RMA-S cells present endogenous antigens to allo-specific CTL. Lysis of untreated RMA (H-2^b, Qa1^b, MTF^a, HMT^a) (circles), RMA-S (squares), *Ham-2*-transfected RMA-S clone Q11 (triangles), and concanavalin A-stimulated lymphoblasts from mouse strain B6.CAS3 (H-2^{cas3}, Qa1^{cas3}, HMT^a) (diamonds) by a, CTL line NZB/Bom anti-MTF^a; b, CTL clone 3E5, specific for Qa1^b; c, H-2^{cas3} anti-H-2^b bulk culture CTL.

METHODS. The NZB/Bom anti-NZB/lbm CTL line is specific for the MTF^a peptide of Mta (ref. 17) and has been described²⁹. Clone 3E5 is an anti-Qa1 CTL clone generated from B10.BR (H-2^k, Qa1^a) anti-C3H/HeJ (H-2^k, Qa1^b) mixed lymphocyte cultures³⁰. Bulk CTL specific for H-2^b antigens were generated in primary mixed lymphocyte culture using B6.CAS3 responder cells and BALB.B stimulators. Targets were labelled with ⁵¹Cr and incubated with CTL for 3.5 h²⁹. Per cent specific lysis was calculated as (experimental release - spontaneous release) × 100/(maximum release - background).



Spontaneous release ranged from 7% to 28% of maximum release. Similar results were obtained in four (Mta) or five (Qa1^b and H-2^b) independent experiments. Addition of a peptide that mimics MTF^a fully restores Mta expression on RMA-S cells³¹.

pathway uses a single functional peptide transporter, consisting of a heterodimer of the *Ham-1* and *Ham-2* gene products. □

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Meiotic initiation by the *mos* protein in *Xenopus*

Nelson Yew, Michael L. Mellini*,
& George F. Vande Woude

ABL-Basic Research Program, and * Protein Isolation Laboratory, PRI/DynCorp Inc., NCI-Frederick Cancer Research and Development Center, PO Box B, Frederick, Maryland 21702-1201, USA

WHEN fully grown *Xenopus* oocytes are stimulated by progesterone, a period of protein synthesis is necessary for maturation¹. Synthesis of the *mos* proto-oncogene product, pp39^{mos}, is necessary for the activation of M-phase promoting factor (MPF) in meiosis I (ref. 2). On the basis that *mos* is translated *de novo* on hormonal stimulation of *Xenopus* oocytes³ and that injecting *mos* RNA into oocytes induces their maturation^{3,4}, we have proposed that the *mos* protein is a candidate initiator of oocyte maturation, needed to trigger the conversion of precursor MPF into its active form^{1,3}. To determine whether *mos* is the only protein required for initiating maturation, we have produced a soluble, active recombinant *mos* protein and injected it into *Xenopus* oocytes. We report here that in the absence of protein synthesis that *mos* protein efficiently induces germinal vesicle breakdown and the activation of MPF. The oocytes, however, do not proceed into meiosis II. Thus, the *mos* protein fulfills the requirements of an initiator protein, but the synthesis of one or more additional proteins may be necessary to complete oocyte maturation.

To generate soluble *mos*, we fused the *Xenopus mos* gene in frame to the *malE* gene of *Escherichia coli*, which encodes maltose-binding protein (MBP)⁵. The vector used for this

construct contains a promoter that is inducible by isopropyl thiogalactopyranoside (IPTG). When this system was induced with IPTG, the MBP-*mos*^{xe} fusion protein represented ~1% of the total protein, and ~50% of the fusion protein was soluble (data not shown). The fusion protein was partially purified to ~90% homogeneity using a two-step procedure (Fig. 1). We also constructed a fusion protein containing a mutant form of *Xenopus mos* (MBP-*mos*^{xekm}), in which lysine 90 of the ATP-binding site was changed to arginine. This mutation abolishes protein kinase activity⁶. MBP-*mos*^{xekm} was expressed and purified in the same manner as MBP-*mos*^{xe}.

The *mos* protein is required not only for the activation of MPF during meiosis I and II (refs 2, 7, 8), but also for the arrest of oocyte maturation at metaphase of meiosis II (ref. 9). Injecting *mos* RNA into one blastomere of a two-cell embryo arrests cleavage of the injected blastomere at metaphase⁹. This and other evidence support the hypothesis that *mos* is an active component of cytoskeletal factor (CSF), which is presumed to be responsible for the normal arrest of maturing oocytes at metaphase of meiosis II (refs 10, 11). Because cleavage arrest of embryos is one of the most sensitive assays for *mos* function¹², we injected the fusion protein into cleaving *Xenopus* embryos, whereupon it efficiently arrested cleavage in a concentration-dependent manner (Fig. 2a). The arrested embryos had high levels of MPF activity (data not shown), indicating that they had been arrested in mitotic metaphase. Injecting ~1.4 ng fusion protein per egg arrested 100% of the recipient embryos, whereas as little as 0.28 ng arrested 40% of the embryos (Fig. 2a). By comparison, roughly 0.2 ng of pp39^{mos} is estimated to be present in the unfertilized egg¹³. Injecting 13.5 ng MBP-*mos*^{xekm} did not induce cleavage arrest, indicating that the arrest was dependent on kinase activity (Fig. 2a).

The purified MBP-*mos*^{xc} protein showed no detectable autophosphorylation *in vitro* (Fig. 2b, lane 5). To determine whether the autophosphorylation activity could be activated *in vivo*, we assayed for kinase activity at different times after injecting the protein into fully grown oocytes. The oocytes were treated with cycloheximide, to prevent endogenous pp39^{mos} expression, and progesterone, to initiate the early events of oocyte maturation¹⁴. After 0, 1 and 2 h, the MBP-*mos*^{xc} protein was immunoprecipitated from the injected oocytes by using a *Xenopus-mos*-specific antibody² and an *in vitro* kinase assay done on the immune complex. Autophosphorylation activity of MBP-*mos*^{xc} was not detected immediately after injection, but low levels were observed after 1 h and the complex was quite active after 2 h (Fig. 2b). The autophosphorylation activity was roughly comparable to that of endogenous c-*mos* in progesterone-matured oocytes (data not shown). The kinase mutant MBP-*mos*^{xekm} was inactive 2 h after injection (Fig. 2b, lane 4). These results indicate that MBP-*mos*^{xc} has kinase activity, but only after being modified in some way *in vivo*.

In the above assays, the oocytes injected with MBP-*mos*^{xc} underwent germinal vesicle breakdown (GVBD) after 2 h (data not shown). This suggested that the fusion protein efficiently induced maturation even when protein synthesis was blocked. We tested the amount of MBP-*mos*^{xc} required to induce maturation in the presence or absence of cycloheximide and progesterone. With 13.5 ng of MBP-*mos*^{xc}, 90% of the oocytes underwent GVBD in the absence of cycloheximide, whereas ~50% of oocytes underwent GVBD in the presence of cycloheximide (Fig. 3). The addition of progesterone to the cycloheximide-treated oocytes increased the frequency of GVBD to

nearly 100%. With 2.7 ng of MBP-*mos*^{xc}, 80% of the oocytes underwent GVBD in the presence of progesterone and cycloheximide, and some activity (~14%) was detected with 0.7 ng of MBP-*mos*^{xc}, roughly the amount of endogenous pp39^{mos} in unfertilized eggs (Fig. 3). The kinase-mutant protein was inactive in this assay, showing that the induction of maturation was due to a specific effect of the kinase-active fusion protein. Progesterone transiently decreases cyclic AMP levels, which leads to a decrease in both protein kinase A and protein kinase C activities¹⁴. These or other biochemical changes that occur on progesterone stimulation seem to enhance markedly the ability of the *mos* protein to induce GVBD.

To determine whether the MBP-*mos*^{xc}-injected oocytes that underwent GVBD were able to exit meiosis I and enter meiosis II, we monitored the levels of MPF activity during and after GVBD. Active MPF phosphorylates histone H1 *in vitro* and this can be used to measure MPF activity¹⁵. As shown previously¹¹, the H1 kinase activity of oocytes induced to mature with progesterone peaked at GVBD, fell to a minimum by 1 h after GVBD, then gradually rose again between 1 and 5 h after GVBD (Fig. 4a). The H1 kinase activity of oocytes injected with MBP-*mos*^{xc} in the absence of cycloheximide displayed a profile similar to that of oocytes treated with progesterone (Fig. 4a). This agrees with previous results⁷ and shows that oocytes induced to mature with *mos* in the absence of cycloheximide do not arrest at meiosis I but proceed into meiosis II. When MBP-*mos*^{xc} was injected in the presence of cycloheximide and progesterone, the first peak of H1 kinase activity was seen at GVBD, but the activity did not increase at later times (Fig. 4a). As a control, we injected *mos*-specific antisense oligonucleotides together

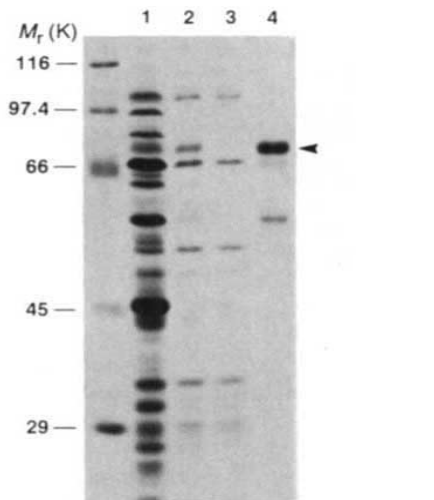


FIG. 1 Purification of a *Xenopus mos* fusion protein from *E. coli*. The samples from each step in the purification were separated by electrophoresis on a 10% SDS-polyacrylamide gel and the gel was stained with Coomassie blue. Lane 1, total soluble protein extract from induced cells; lane 2, Q-Sepharose column flow-through; lane 3, amylose column flow-through; lane 4, amylose column eluate. The arrow indicates the position of the MBP-*mos*^{xc} protein, which has a relative molecular mass of 77,000 (M_r 77 K). M_r markers (in K) are indicated.

METHODS. A 1.1-kilobase (kb) *SacI*-*XbaI* fragment containing the *mos*^{xc}-coding region² was subcloned into the *XbaI* site of pMALC-RI (New England Biolabs) and used to transform *E. coli* TB1 cells. Transformed cells were induced with 0.4 mM IPTG, then lysed with 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mg ml⁻¹ lysozyme. The extract was sonicated for 2 min at 2 °C, then clarified by centrifugation at 9,000 g for 20 min. The supernatant was applied to a Q-Sepharose column (Pharmacia), which does not bind the fusion protein, but does bind many of the host proteins. The flow-through was collected and applied to an amylose affinity column, which specifically binds the MBP portion of the hybrid protein. The column was then washed with 88 mM NaCl, 20 mM HEPES, pH 6.8, 7.5 mM MgCl₂. The protein was eluted in the same buffer containing 10 mM maltose, then dialysed against 88 mM NaCl, 20 mM HEPES, pH 6.8 and stored at 4 °C.

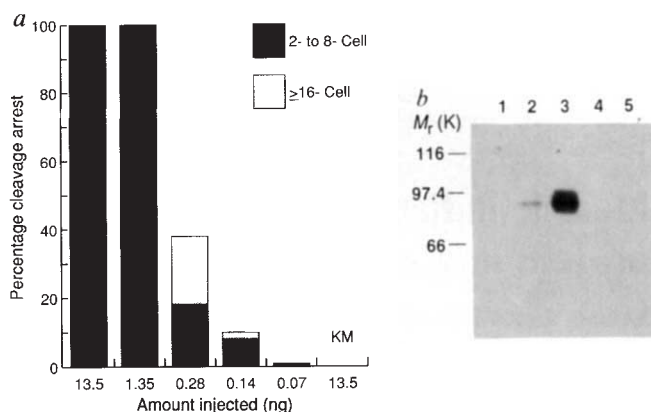


FIG. 2 a, Cytostatic factor activity of MBP-*mos*^{xc}. Dilutions of MBP-*mos*^{xc} were injected into one blastomere of a two-cell embryo. After 6–8 h, the embryos were scored for the stage of cleavage arrest. As a control, injection of MBP-*mos*^{xekm} (KM) was included. b, Autophosphorylation of MBP-*mos*^{xc} *in vitro*. Lanes 1–4, *in vitro* kinase assays of MBP-*mos*^{xc} and MBP-*mos*^{xekm} immunoprecipitated from injected oocytes. Lane 1, 0 h after injection with MBP-*mos*^{xc}; lane 2, 1 h after injection with MBP-*mos*^{xc}; lane 3, 2 h after injection with MBP-*mos*^{xc} (GVBD); lane 4, 2 h after injection with MBP-*mos*^{xekm}; lane 5, uninjected MBP-*mos*^{xc}.

METHODS. Ovulated eggs were obtained and fertilized *in vitro* as previously described⁹. The fertilized eggs were de-jellied with 2% cysteine, then incubated in 0.3× modified Barth's solution containing 5% Ficoll⁹. The fusion proteins were diluted in 88 mM NaCl, 20 mM HEPES, pH 6.8, 1 mg ml⁻¹ bovine serum albumin, and 45 nl was injected into one blastomere. An average of 30 embryos was injected for each dilution. For the *in vitro* kinase assays, each oocyte was injected with 22.5 ng of the indicated fusion protein, then incubated in L-15 medium²² (50% Liebovitz L-15 medium with 100 µg ml⁻¹ gentamicin) containing cycloheximide and progesterone (both at 10 µg ml⁻¹). At each time point, 10 oocytes were lysed in 200 µl of lysis buffer (150 mM NaCl, 10 mM NaPO₄, pH 7.2, 1 mM EDTA, 0.1% Nonidet-P40, 10 µg ml⁻¹ leupeptin). The homogenate was centrifuged at 8,000g for 5 min, and the clear cytosol was collected. Immunoprecipitations using anti-*mos*^{xc} monoclonal antibody (5S) (ref. 2) and *in vitro* kinase assays were done as previously described¹². For the uninjected sample, 225 ng MBP-*mos*^{xc} was diluted in 200 µl of lysis buffer, then immunoprecipitated as above.

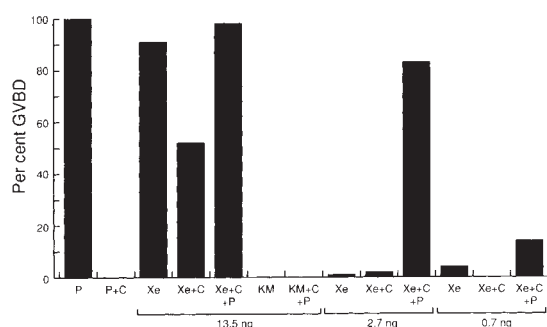


FIG. 3 Induction of GVBD by MBP-*mos*^{xe} in the absence of protein synthesis. Oocytes were injected with the indicated amounts of MBP-*mos*^{xe} then scored for GVBD 6–8 h later. Xe, MBP-*mos*^{xe} protein; C, cycloheximide; P, progesterone; KM, MBP-*mos*^{xekm} protein.

METHODS. Oocytes were preincubated in 50% L-15 medium $\pm$ cycloheximide ($10 \mu\text{g ml}^{-1}$) for 1 h, then injected. The injected oocytes were placed in 50% L-15 medium $\pm$ cycloheximide and progesterone (both at $10 \mu\text{g ml}^{-1}$). After 6–8 h, the oocytes were scored for GVBD by observing a white spot on the animal pole and by fixing the oocytes in 10% trichloroacetic acid for 30 min, then dissecting them to determine the integrity of the nucleus. Each percentage is based on the injection of 60 oocytes.

with MBP-*mos*^{xe}. The oocytes matured and arrested at metaphase of meiosis II, indicating that MBP-*mos*^{xe} can substitute for the endogenous pp39^{mos} during meiosis II (data not shown). Western blot analysis showed that the injected MBP-*mos*^{xe} was not degraded at meiosis I, because the level of MBP-*mos*^{xe} remained roughly the same before and after GVBD (Fig. 4b).

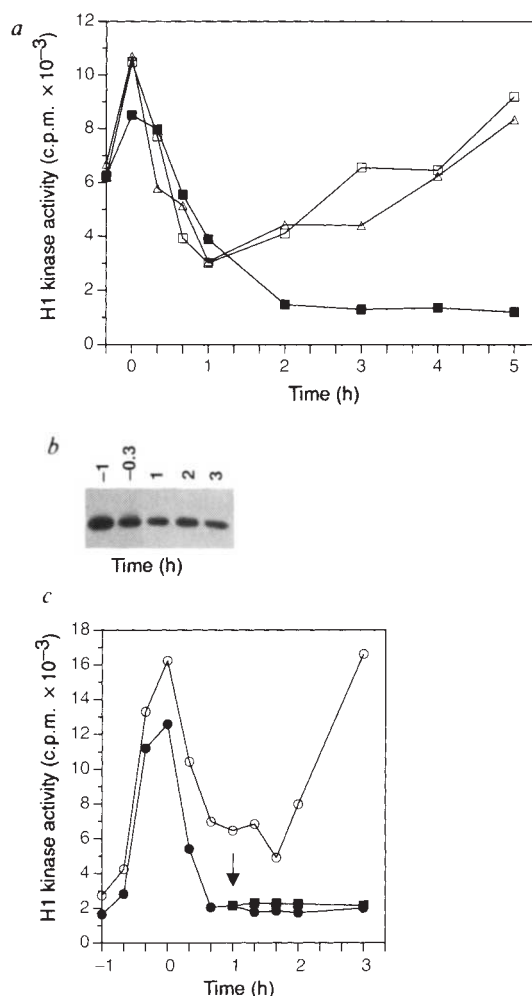
FIG. 4 H1 kinase activity in oocytes after GVBD. *a*, Oocytes were treated with progesterone (Δ) or injected with 22.5 ng MBP-*mos*^{xe} in the presence ($\blacksquare$) or absence ($\square$) of progesterone and cycloheximide. Oocytes were assayed for H1 kinase activity at the indicated times during and after GVBD, with the maximum level of H1 kinase activity designated as 0 h. *b*, Western blot of oocytes injected with MBP-*mos*^{xe}. The cytosols of the injected oocytes were analysed for the relative levels of MBP-*mos*^{xe} at the indicated times before and after GVBD. *c*, Oocytes were injected with MPF (to induce the oocytes to undergo GVBD synchronously) and assayed for H1 kinase activity as in *a*. A portion of the cycloheximide-treated oocytes was reinjected with 22.5 ng MBP-*mos*^{xe} 1 h after GVBD (indicated by the arrow). $\circ$, MPF alone; $\bullet$, MPF in the presence of cycloheximide; $\blacksquare$, MPF plus MBP-*mos*^{xe} in the presence of cycloheximide.

METHODS. Oocytes were incubated in 50% L-15 medium plus $10 \mu\text{g ml}^{-1}$ cycloheximide 30 min before injection. At the indicated times, groups of four oocytes that had undergone GVBD within 10 min of each other were collected and homogenized in 50 μl of EB buffer (80 mM β -glycerophosphate, 20 mM EGTA, 15 mM MgCl_2)¹¹. The homogenate was centrifuged for 4 min in a microcentrifuge, and the cytosol was frozen at -70°C . H1 kinase assays were done as described²³. In some instances, cycloheximide was added 0–1.0 h after GVBD with identical results (not shown). For the protein blots, 15 μl cytosol (equivalent to about one oocyte) from the indicated time points was subjected to electrophoresis on an SDS-polyacrylamide gel and blotted to nitrocellulose. The blot was probed with an anti-*mos*^{xe} mouse monoclonal antibody (5S)² and ^{125}I -labelled goat anti-mouse IgG antibody (Boehringer Mannheim). Exposure time was 42 h. MPF was prepared by lysing progesterone-matured oocytes with two volumes of EB buffer. The homogenate was centrifuged for 4 min in a microcentrifuge, and the cytosol was used for the injections.

The fusion protein remained kinase active after GVBD (data not shown; see also Fig. 2b, lane 3). In other experiments we injected the protein 1 h after GVBD when MPF activity was low. The injected MBP-*mos*^{xe} did not induce the second rise in H1 kinase activity in the presence of cycloheximide (Fig. 4c). The results suggest that, in addition to *mos*, synthesis of other proteins is needed for the successful initiation of meiosis II.

As first described by Wasserman and Masui, the synthesis of one or more proteins is required for the G₂-M transition during the cycloheximide-sensitive period immediately after progesterone stimulation of *Xenopus* oocytes¹. Until recently, cyclin was thought to be one of these proteins, because cyclin synthesis is both necessary and sufficient to drive the early mitotic cycles of the embryo^{16,17}. But the oocyte contains stockpiles of B-type cyclin proteins with a portion already complexed to p34^{cdc2} (pre-MPF), and oocytes are still able to respond to progesterone and proceed through both meiosis I and II even when all cyclin RNAs are first destroyed using antisense oligonucleotides^{18–20}. Thus, it seems that the synthesis of a protein or proteins other than cyclin is necessary for entering meiosis I. Previous results showing that *mos* synthesis is required for maturation², together with our results, suggest that *mos* is both necessary and sufficient for the initiation of meiosis I.

The synthesis of *mos* is required for meiosis II in *Xenopus*^{7,8} and our results suggest that the synthesis of other proteins is also needed. Injecting active MPF into oocytes almost immediately increases the rate of protein synthesis²¹, which suggests that the M-phase activity of meiosis I could trigger the translation of the proteins necessary for meiosis II. Potential candidates are proteins that would interact with *mos* to induce M-phase arrest. Such proteins could account for why oocytes do not



arrest in meiosis I, even though *mos* is present during this period. □

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Inside polyomavirus at 25-Å resolution

James P. Griffith*, Diana L. Griffith*, Ivan Rayment*†, William T. Murakami‡ & Donald L. D. Caspar*§

* Rosenthal Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254, USA

‡ Graduate Department of Biochemistry, Brandeis University Waltham, Massachusetts 02254, USA

EMPTY capsids and complete virions of polyomavirus crystallize isomorphously¹. Here we use difference Fourier analysis of X-ray diffraction data at 25-Å resolution from these crystals to obtain an electron-density map of the inside of the virion. The polyomavirus capsid is built from 72 pentamers of VP1 that form three different types of connections in the $T=7d$ icosahedral surface lattice². Self-assembly of purified recombinant VP1 into capsid-like aggregates^{3,4} has shown that switching of the bonding specificity to form the unanticipated⁵ non-equivalent connections is an inherent property of the VP1 pentamers. Our map of the inside of the virion displays 72 prongs of electron density extending from the core into the axial cavities of the VP1 pentamers. We identify these prongs with the VP2 and VP3 molecules, which may function to guide the assembly of the highly ordered capsid on the nucleohistone core. The atomic structure of the closely related simian virus-40 capsid has been determined from the high-resolution diffraction data⁶. Our polyomavirus map, calculated using all the low-resolution diffraction data, shows no indication of regular order inside the spherical core.

Diffraction data from virion crystals were collected by screened precession photography, in the resolution range 200–22.5-Å spacing, for comparison with previously analysed capsid data^{2,7}. Intensities for the three unrecorded lowest order virion reflections and F_{000} were calculated by iterative interpolation using the solvent-flattening restraint⁸. Below 50-Å resolution,

there are substantial differences between the virion and capsid data due to diffraction from the inside structure, but at higher resolution the two data sets are similar. To compute a difference map, the two data sets were scaled over the range 0.02–0.044 Å⁻¹ by applying an adjustable temperature factor to account for the somewhat greater disorder in the capsid crystals. The capsid preparation² consisted predominantly of VP1 with only trace amounts of VP2/VP3. On the approximation that the effective number of scattering electrons in the virion and capsid are proportional to their masses, F_{000} for the virion was scaled to that for the capsid by the ratio of their relative molecular masses. The relative molecular mass (M_r) of an empty capsid, which consists of 360 VP1 molecules², is 15.3×10^6 . The complete virion⁹ includes the minor viral proteins VP2 and VP3 (M_r of 35,000 and 23,000 (35K and 23K) respectively) and the 3,300K circular double-stranded DNA molecule complexes with cellular histones. Data from analyses of the nucleosome¹⁰ and minor protein¹¹ compositions of the closely related SV40 and polyomavirus⁹ indicate that the virion has an M_r of $23,500K \pm 3\%$.

Electron-density maps of the virion and capsid (Fig. 1a, b) were computed at 25-Å resolution from the scaled structure factors and phases that were refined, starting with those previously obtained^{2,7} for the capsid structure. The phases for the virion and capsid were subjected to 18 cycles of real space refinement consisting of icosahedral symmetry averaging and solvent flattening outside an envelope whose contour loosely followed the particle surface. For the empty capsid, the envelope was ~10 Å wider than that just enclosing the volume of the 360 VP1 molecules; for the virion, only the outside of this loose envelope was applied in the refinement. The final R factors comparing the transforms of the solvent-flattened, symmetry-averaged virion and capsid maps with the corresponding data were 0.103 and 0.095, respectively. The fluctuations in the difference map (Fig. 1c) within the capsid envelope are comparable to the noise level in the solvent volume, which demonstrates the reliability of the phasing and scaling. Because the difference map was calculated using all the low-resolution data and an appropriately scaled F_{000} , the mass distribution of all the inside structure can be quantitatively evaluated.

Surface representations of the virion, capsid and difference maps (Fig. 1d–f) indicate how the spherical core is connected to the capsid by the 72 prongs. The core is separated from the bases of the pentameric capsomeres by regions of low density (Figs 1a and 2), as was observed in the 35-Å-resolution reconstruction of the SV40 virion structure by cryoelectron microscopy¹². The overall shape and mass distribution of a prong are well defined by the symmetry-averaged difference map (Fig. 2). Scattering matter in the portion of the prong nestled in the VP1 pentamer cavity is packed more densely than near the core, indicating more ordered structure at the tip. Localized density in the same region on the pentamer axis has been found in the high-resolution electron density map of SV40 (ref. 6), which has been identified as a segment of VP2 or VP3.

Evidence for specificity in the interaction between VP1 and the minor capsid proteins implies that the prongs connecting the VP1 pentamers to the core consist of some portion of the VP2/VP3 molecules. Empty capsids, isolated from infected cells, lack detectable nucleohistone but generally contain residual amounts of VP2/VP3, sometimes approaching the normal virion content (W.T.M., unpublished results), indicating a preferential association of the minor proteins with VP1. Furthermore, interaction between recombinant VP1 pentamers and VP3, which requires the carboxyterminal 40-amino-acid segment of VP3, has been demonstrated *in vitro*¹³.

The correspondence between the number of prongs (72) and the estimated number of VP2 plus VP3 molecules per virion ($(21 + 56) \pm 10\%$, as evaluated from scaling the SV40 minor protein bands to the VP1 component on SDS-polyacrylamide gels¹¹) indicates that each prong represents just one minor protein molecule. VP3 and the C-terminal two thirds of VP2 are coded

† Present address: Enzyme Institute, University of Wisconsin, Madison, Wisconsin 53705, USA.

§ To whom correspondence should be addressed.

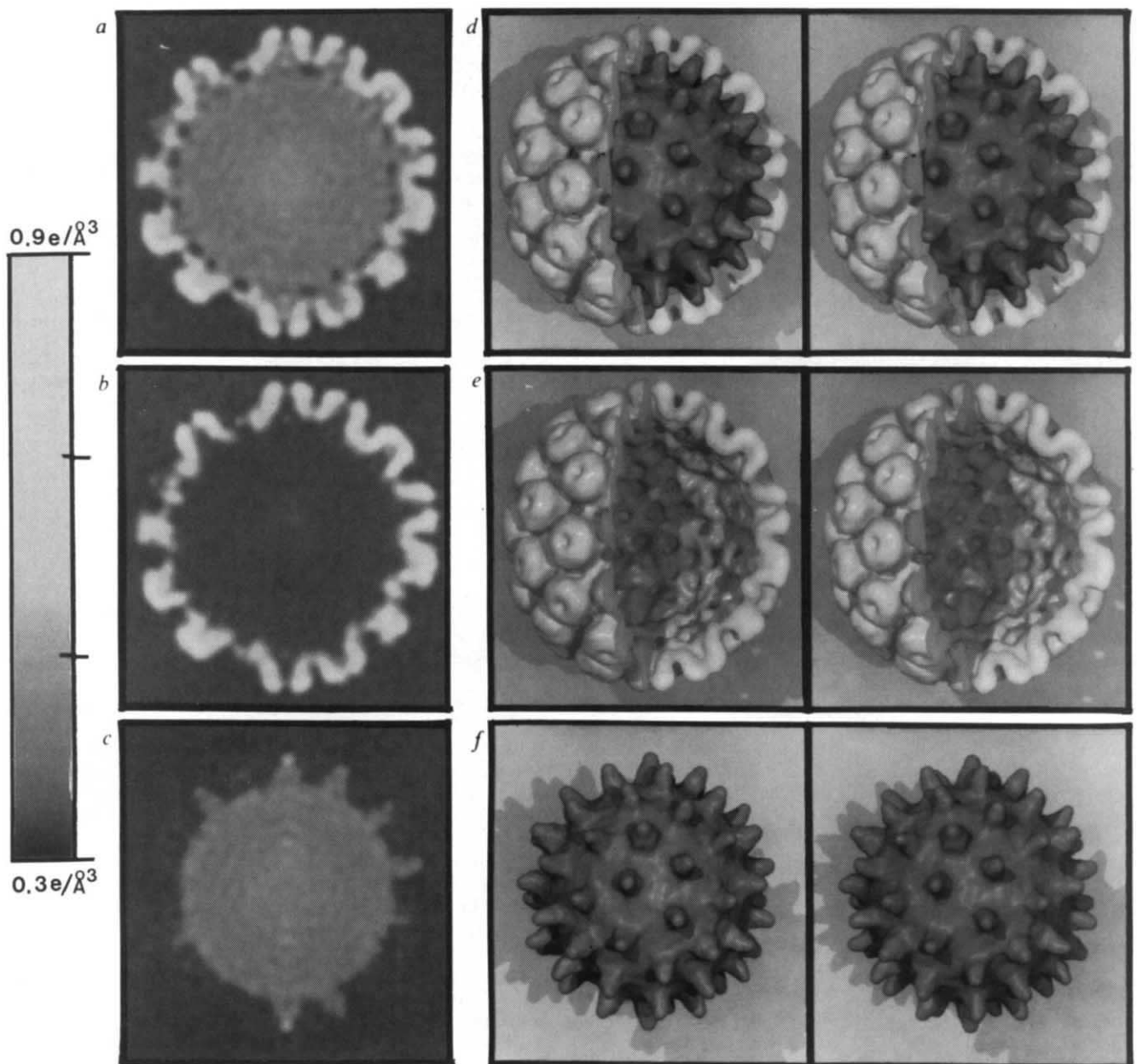


FIG. 1 Sections (a–c) and three-dimensional stereographic surface representations (d–f) of the electron-density maps of the virion (top), the capsid (middle) and the virion-capsid difference (bottom). The sections a–c slice through the centre of the particles and the view is perpendicular to both an icosahedral 5-fold axis (running vertically) and a local hexavalent pentamer axis (22° to the right of the icosahedral 5-fold axis at the upper part of the sections). The outer diameter of the capsid measured along a 5-fold axis is 500 Å. The grey scale to the left defines the electron density levels in the maps. Sections a–c are from the icosahedrally averaged maps

computed using the measured structure factors with the refined phases. The three-dimensional surface display of the capsid map (e) was contoured to contain a volume equal to that calculated from the mass and partial specific volume of the 360 VP1 molecules. In d and e, a 108° sector was removed from the VP1 capsid structure, such that the cut surface is equivalent to the section of the capsid in a and b. The surface contour for the difference map (f) was set such that the prongs projecting from the core nestled inside the axial cavities of the VP1 pentamers in the virion map (d).

by the same DNA sequence⁹. Deletion mutants of SV40 lacking VP2 were found to reproduce slowly, but viable mutants without VP3 were not detected¹⁴. Thus, the N-terminal third of VP2 is not required for viral assembly, but some portion of the VP3 sequence seems to be essential. We conclude that the prongs comprise the part of VP3 or the equivalent part of VP2 involved in connecting the VP1 pentamers to the nucleohistone core.

How much protein is in the connecting prongs has been estimated by integration of the electron density distribution in our difference map (Figs 1c and 2). In the hexavalent and pentavalent positions, the mass-per-prong domain (as

delineated in Fig. 2) is 14.8K and 18.9K, respectively, but the difference in these values is within the uncertainty of the measurements. The mean mass of a 50-Å-long prong domain (15.5K) accounts for about two thirds of a VP3 molecule. Although electron density carries no chemical label, the correlation of biochemical and structural data indicates that the prong domain corresponds to a major portion of the VP3 sequence. Similarly, identification of the spherical core with an irregular packing of the ~26 nucleosomes of the minichromosome is consistent with data on the nucleohistone structure^{9,10,15}.

The core density distribution appears spherically symmetric

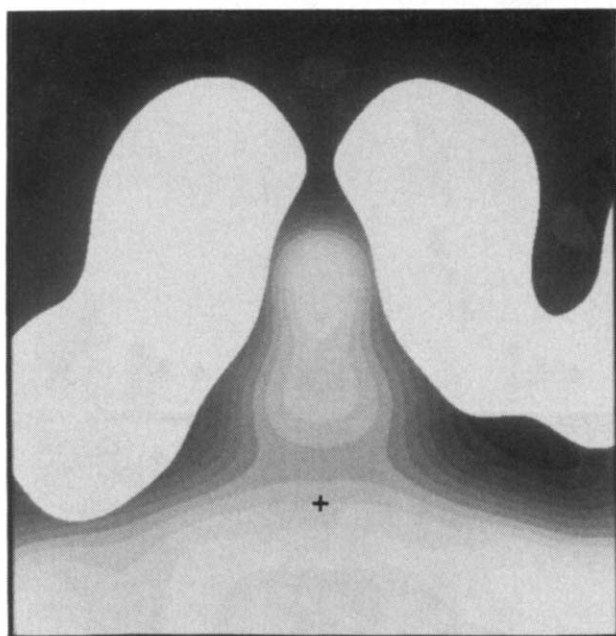


FIG. 2 Section of the symmetry-averaged difference map of a prong inserted in the outline of a VP1 capsomere. Five sections related by the local hexavalent pentamer axis and one corresponding section of a pentavalent pentamer (oriented as in Fig. 1c) were averaged to generate this map. The section of the prong map, displayed on a 12-step grey scale, is contained inside the outline of the capsid envelope as contoured in Fig. 1e. The step interval of the grey scale is 0.0065 electrons/Å³, which corresponds to the r.m.s. noise level measured from the fluctuations in the solvent volume of the electron-density map. The figure frame is 120-Å square and the pentamer axis is vertical at the middle. The plus sign marks the level defined as the boundary between the core and the prong, which is at radii of 174 and 170 Å on the pentavalent and hexavalent pentamer axes, respectively.

within the noise level measured from the solvent portions of the maps (Fig. 1a-c). At the centre, the core density is higher than near its surface, as shown by the spherically averaged density profiles (Fig. 3). The measured density distribution can be accounted for by about four nucleosomes that occupy the centre of the core out to ~85 Å radius, and a mean of ~22 nucleosomes, less compactly packed, in the shell from ~85-170 Å radius. Although there is no evidence of any icosahedral order within the core, there must be some type of local order among the closely packed nucleosomes which varies from virion to virion

in the crystals. The liquid-drop model for the isolated compact minichromosome, inferred from cryoelectron microscopy¹⁵, may also represent the irregular packing of the nucleosomes in the virion.

Capsid proteins of the DNA tumour viruses are synthesized in the host cell cytoplasm and are then transported to the nucleus where they assemble with the viral minichromosomes to form the icosahedral virus particles⁹. VP1 molecules expressed in insect cells are also transported to the nucleus where the pentamers self-assemble into capsids independently of the minor capsid proteins and minichromosome¹⁶. In normally infected cells, association of VP1 pentamers with the common C-terminal portion of VP2 or VP3 can occur in the cytoplasm¹³ or in the nucleus, because both VP1 and the minor protein have their own nuclear targeting sequences¹⁷. Insertion of only a single minor protein segment in the pentagonally symmetric cavity can be accounted for by the steric restraints. The extended N-terminal third of VP2, which is not required for viral assembly¹⁴, may form a flexible arm holding the terminal myristyl group, which seems to enhance the efficiency of viral entry into the host cell¹⁸. The essential function of VP3 and the corresponding part of VP2 is apparently to direct the self-assembly of VP1 pentamers on the nucleohistone core, thereby insuring formation of the complete virion. □

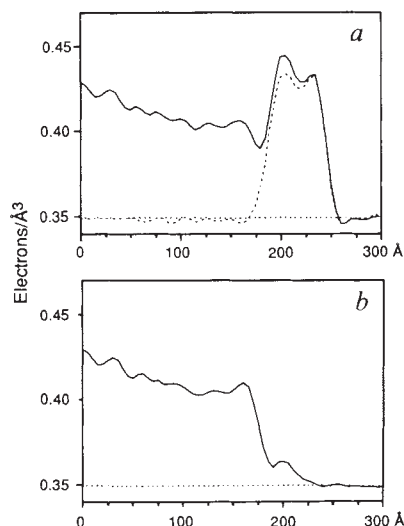


FIG. 3 Spherically averaged electron-density profiles of *a*, the capsid (dashed curve) and virion (solid curve) and *b*, the difference between them. These spherical averages were computed from the three-dimensional maps, which were scaled according to the effective number of scattering electrons per unit cell, with the solvent density of 0.35 electrons Å⁻³. The virion and capsid profiles superimpose from about 226 to 250 Å radius. From 0-226 Å radius, the difference (*b*) between the virion and capsid profiles maps the radial distribution of the matter inside the virion. The virion profile shows a region of low density at around 175-Å radius, which indicates the boundary between the core and the bases of the capsomeres. The ripples in the core region of the profiles are the order of the noise fluctuations in the spherically averaged density measurements.

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Software sourcebook

Whether you are hooked on the Apple Macintosh or personal computers, this week's feature has something to suit both camps. Featured products include programs for three-dimensional reconstruction and DNA/protein band matching, and software that is designed to take the strain out of grant writing.

MINNESOTA Datametrics announces the release of *ImageVolumes*, an **image processing and three-dimensional reconstruction software system** for Silicon Graphics' 4D Series and Indigo workstations (*Reader Service No. 100*).



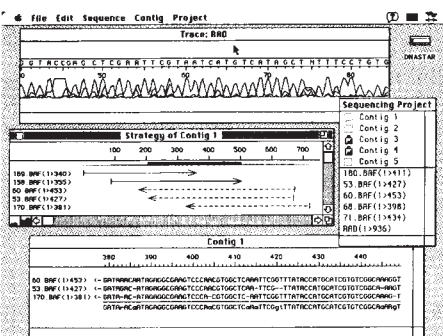
Shaded surface rendering of a three-dimensional reconstruction of human brain showing the external limiting dura mater. (Courtesy of Magnetic Resonance Research, University of Minnesota, Minneapolis, Minnesota, USA.)

Input to *ImageVolumes* can be digitized grey-scale images or graphics data describing contours and points. Interactive editors are included for both forms of data. Output includes high-quality, shaded-surface renderings, and morphometric analysis, including numbers of objects, surface areas and volumes. The cost of the *ImageVolumes* package for all Silicon Graphics' workstations is \$8,125 (US).

The new **fractal analysis program** from Seescan offers a form of shape analysis and has been designed to allow numerical definition of objects with complicated shape, which show self-similarity when viewed at different levels of magnification (*Reader Service No. 101*). Users will be able to use the package to assess the directionality and branching of dendritic growth from neurons. The fractal dimension also can be used to provide a mathematical assessment of the 'shape' of a suitable object such as a skin lesion or portion of polymeric material, Seescan says. Designed for use with the company's range of fully-automated video image analysis systems, the program uses three different methods of approximating the fractal dimension of an imaged object: the Richardson's perimeter walking method, the box-counting method and the dilation method.

OLIGO version 4.0, a **primer selection and analysis program** from National Biosciences, is now available for Apple Macintosh computers (*Reader Service No. 102*). OLIGO will search GenBank, EMBL, ASCII and most other DNA/RNA sequence files for optimized primers (PCR), sequencing and hybridization applications. The program selects highly specific, stable, unique, dimer and hairpin-free oligonucleotides within a user-specified range. Using 'nearest neighbour' thermodynamic algorithms, OLIGO also provides accurate calculations for optimal annealing temperature and primer/product Tms and Tds. In the analysis mode, OLIGO displays instant updates of dimer/hairpin structure, false priming sites, 3' terminal stability and composition of various oligonucleotides of interest on the sequence file. The 3' terminal stability calculations ensure the selection of high specificity ('single band') PCR and sequencing primers, the company says. Fully automated searches and 'HELP' functions throughout OLIGO facilitate its use.

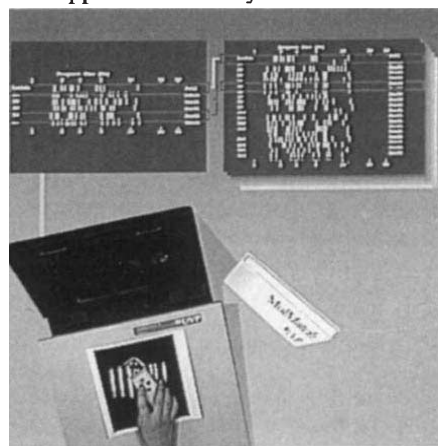
According to DNASTAR, its SeqMan II **assembly software** for the Apple Macintosh can assemble full cosmid projects from hundreds of sequences as easily as a single cDNA (*Reader Service No. 103*). SeqMan II uses an advanced two-pass



Now in Mac format — SeqMan II assembly software from DNASTAR.

algorithm that takes account of the declining quality of data at the ends of gel reads. The program also eliminates many problems caused by repetitive DNA elements. Taking full advantage of the graphical capabilities of the Macintosh, users can even display trace data from the popular fluorescent sequencing machines directly on screen while editing consensus alignments, DNASTAR says.

MolMatch, which was developed through the collaborative efforts of Ultra Violet Products and Glasgow University, is a software package that allows the user to **size, store and manipulate DNA or protein data from electrophoretic gels** (*Reader Service No. 104*). The software features pattern matching facilities used in conjunction with user-created 'fingerprint' libraries; this, UVP says, should find application in many fields of research.



Matchmaking with UVP's MolMatch.

Gel images are digitized and stored. MolMatch can store up to 2,000 tracks in each library. To speed comparisons, each user can build composite files (of up to 400 tracks) from the stored data, selecting each track by name and specifying its position in the file. New data can be added to the composite file, which then can be displayed on the screen, numerically or graphically, to allow comparisons. MolMatch can search its library for tracks matching a selected sample to within a specified percentage of fragment size variation. The software complements UVP's GDS2000 gel documentation system, although its use is not restricted to it alone. MolMatch runs on IBM and compatible computers and is supplied with a digitizing tablet.

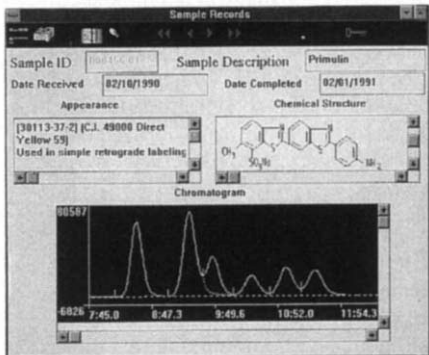
Applications software

The new **V-200 materials testing software package** from Liveco is designed specifically for use with the Vitrodyne materials tester, an instrument that evaluates the physical properties of a wide variety of biomaterials (*Reader Service No. 105*). The V-200 testing program features quick set-up and implementation of standard material tests such as stress-strain, load displacement, creep and recovery,

stress relaxation and monitoring continuous cyclic tests. Data are logged automatically at user-specified rates, while test results are displayed in real-time on the computer monitor. Post-test analyses include maximum-minimum values, line slope and area under curves.

VG Data Systems will be previewing the new version of Multichrom 2, a **chromatography data system for the VAX**, next month at the Pittsburgh Exposition and Conference to be held in New Orleans (*Reader Service No. 106*). This latest release offers new advantages to the chromatographer through features such as the enhanced instrument control option. By using this facility, the operator has the ability to control a wide range of instruments from different vendors, and it allows the end user to define the parameter set to be loaded into the instrument, the layout of the screen(s) for those parameters and the protocol utilized when communicating with each unit. Operators now have a choice of three GLP validation toolkit options on Multichrom 2. In addition to these new options, Multichrom 2 has been further enhanced to include extra graphical manipulation of calibration curves, the capability to discard outlying calibration points and review the subsequent curve with a display of full statistical information.

SKYLIGHT is a **Windows-based database package** from Harley Systems that has the ability to store pictures as database fields and incorporates a graphics report generator that allows reports to be designed in any format (*Reader Service No. 107*). All records can be stored in conjunction with high-resolution im-



ages (chromatograms, photographs, charts and diagrams). This, Harley claims, makes the package particularly suitable as a laboratory database system and eliminates the need to store chromatograms in paper form separately from the rest of the document. Chromatograms input by a scanner or video camera can be stored as an image field within the database as part of the document. The document can then be referenced and retrieved by any of the other fields that make up the document,

such as name, date, ID or password. Being Windows-based, SKYLIGHT is able to share the same clipboard with most modern spreadsheets, word processing, graphics and desktop publishing packages. This facilitates the transfer of data between systems — a prerequisite for any database package, Harley says. SKYLIGHT is intrinsically multi-user, having been designed for use on LANs, but it works on single-user PCs as well.

Dynatech Laboratories has introduced new **grain counting software** for use with the company's SAMBA 4000 cell image analysis system (*Reader Service No. 108*). The software is designed specifically for grain counting, *in situ* hybridization of radioactive probes, and DNA repair assessment by means of tritiated thymidine. With applications in pathology, cytology and neuroscience, the software can be used to count silver staining grains in nuclear organizing regions as markers of cell proliferation activity, to describe micronuclei, and to determine the distribution of neuroreceptors with DNA, mRNA and peptide probes. When making a determination of numbers of grains, the software includes a correction for overlapping grains in high-density areas using the mean grain area. Adaptive thresholding allows the system to distinguish grain from nuclei regardless of variations of counterstain within the cell, Dynatech says. Printed reports display histograms that describe frequency of grains/nuclei in relationship to nuclear area and calculate the ratio of mean grain area to nuclear area.

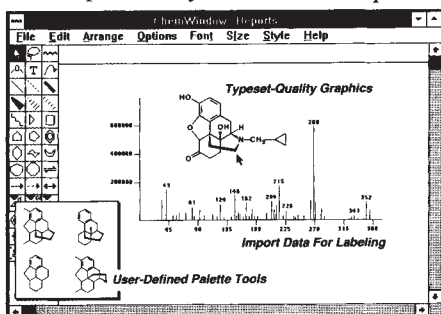
Version 3.0 of ELISA+, **data reduction software for microplate readers**, is now being shipped by Meddata (*Reader Service No. 109*). Major program enhancements for this latest version include a fluorescence reader interface and specialized features for laboratories that use antibodies for ELISA/EIA test development, kit production and basic research. Perhaps the most important feature of ELISA+ 3.0 is the ability to obtain titres from sets of dilution curves. The titration point for the curves can be fixed or calculated automatically relative to the range of absorbance values in a specified area on the plate. Coupled with dilution curves, is a graphical display and printout of up to 12 curves on one plot. Similarly, parallelism between curves also can be determined by the graphics functions of ELISA+ 3.0.

Quick on the draw

The new **two-dimensional drawing tool**, which is now included as part of the latest release of Chemical Design's Chem-X molecular modelling software program, is designed to enable chemists to draw molecules onscreen in the same way that

they would on paper (*Reader Service No. 110*). Molecules are built up from rings, chains and elements selected from the 2D drawing panel enabling laboratory chemists to draw molecules of drugs in less than 15 seconds and complex steroids in under a minute, the company says. The sketches can then be converted to three-dimensional coordinates for further studies using the visualization, computation and database facilities of Chem-X. The basic fragments available for 2D drawing include both saturated and unsaturated five- and six-membered rings. Rings may be joined by spiro or bond fusions. Chains are then added to the basic ring system, initially as carbons (without hydrogens), which may then be changed to other atom types. Bond order can be altered simply by picking a bond, the company says. When the molecule is complete, the 2D-3D converter automatically adds hydrogens and produces the 3D representation. Chem-X is available in PC, Apple Macintosh, UNIX workstation and VAX formats.

ChemWindow version 2.0 is a **chemistry drawing program** from SoftShell designed to create typesetter-quality graphics for chemical reports and papers (*Reader Service No. 111*). Version 2.0, a major upgrade of the original ChemWindow, has more than 40 new features, including multiple drawing styles, a built-in scrapbook, style-sensitive templates,



Chemistry graphics software for Windows. Improved text handling and the ability to read and edit Macintosh documents created by ChemWindow's sister product, ChemIntosh. Users can create multiple drawing styles, such as small and light graphics for reports, or big and bold graphics for presentations. Other features of ChemWindow include: tools for rings and bonds, an acyclic chain tool that draws in any direction, Bézier curves and arrows, Lewis dots, circled charges, multiple 'undo' commands, one-electron arrows, orbitals, rulers, align and space commands, a reduced view option, and oval and arc tools. The package is compatible with all Windows applications, with WordPerfect and with most DOS applications. ChemWindow version 2.0 costs \$499 (US).

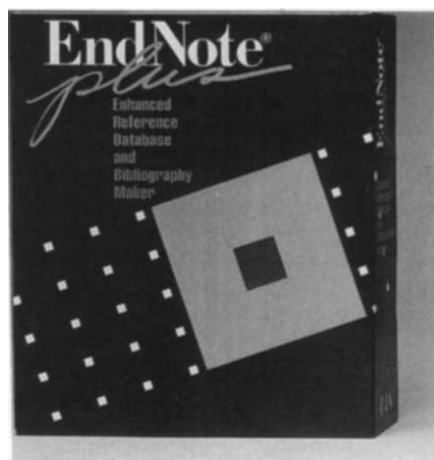
Desktop Molecular Modeller (DTMM) version 2.0 is a **molecular modelling**

package from Oxford University Press that brings construction, manipulation and calculation techniques to microcomputer users (*Reader Service No. 112*). With DTMM, users can construct molecules with an atom limit of 2,700 (3,400 for the maths co-processor version), view them in a variety of styles and orientations, edit and validate structures, carry out a range of calculation techniques, and save or print the results. DTMM uses forcefield calculations to minimize energy with respect to the following: bond stretching, bond angle bending, torsion angle rotation, and non-bonded energies including Coulombic forces and hydrogen bonding. About 30 structures and fragments are supplied with the program, with additional structure libraries and programs available that further extend the scope of the program. DTMM for IBM PCs and compatible computers is priced at £295 (UK).

Time-saving toolkits

Is grant writing a chore? Then, Research Information Systems thinks it may have the answer. The company is now shipping version 2.0 of Grant Accountant, an upgraded software package that runs on MS-DOS personal computers, which is designed to assist researchers and academic departments in **administering and monitoring the status of their grant funds** (*Reader Service No. 113*). Grants can be broken down into any number of subaccounts, including employee salaries and benefits. Purchase order requisitions can be generated and printed from vendor catalogues, and the funds automatically encumbered from the specified grant(s). Reports on the status of grants, sub-accounts, orders, and employee transactions can be viewed on the screen or printed. Several enhancements have been added to version 2.0, including the entry of more detail about grants, subaccounts, vendors and their catalogue items, and employees. Another new feature is intended to allow the researcher to reconcile quickly and easily the institution's reports against the information in Grant Accountant, and to make any necessary adjustments.

EndNote Plus is a new enhanced version of Niles and Associates' **bibliographic software** (*Reader Service No. 114*). Of special interest in this version are the new QuickFind feature and the program's ability to read and write Microsoft Word for Windows documents. EndNote Plus stores up to 32,000 records and can generate bibliographies in many journal styles. The user can 'paste' or type citations from their database into their word processor document. Then, EndNote Plus scans the finished paper for in-text citations and reformats it according to the desired journal style, adding a reference



Reference organizer for the PC.

list at the end. The company says that the QuickFind feature (a full text index) allows users to find records quickly, even in a file with thousands of references. EndNote Plus also addresses the need to find duplicate records, a problem that can arise when importing records from colleagues or online services. Niles has also tackled the problem that arises when different journals follow different abbreviation standards for references. EndNote Plus costs \$249 (US), although EndNote (for the PC) users can upgrade for \$49 (US).

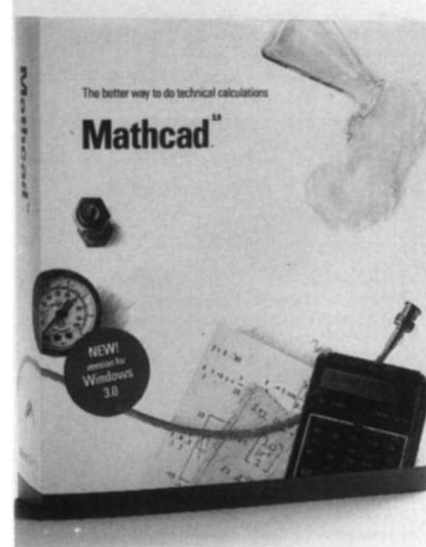
SlideWrite Plus for Microsoft Windows 3.0, **technical presentation software** developed specifically for scientists by Advanced Graphics Software, combines a WYSIWYG (what-you-see-is-what-you-get) user interface with onscreen control of graphs, text and drawings (*Reader Service No. 115*). Scalable Nimbus-Q fonts (including Greek and maths symbols) and vector-format clip art that ensure the integrity of both text and drawings is maintained, regardless of the size of the output, the company says. SlideWrite Plus charts can easily be moved via the Windows clipboard for inclusion in WordPerfect, Word, PageMaker or Ventura for Windows documents. The program accepts data from Lotus files, ASCII files, and from any application using dynamic data exchange such as Excel or QuattroPro. Over 400 logically grouped, vector-format illustrations of objects, arrows, chemical symbols, plants, animals, flow charts, scientific apparatus and symbols etcetera are provided with the package. SlideWrite Plus for Windows costs \$445 (US).

Problem solving

MicroMath Calc (MMCalc) is a **desk accessory calculator** from MicroMath for the Apple Macintosh (System 7 version) that offers not only the standard mathematical and financial functions of most calculators, but also many advanced features that, to date, have not been available

on the Mac (*Reader Service No. 116*). Designed specifically for scientists, programmers, mathematicians and engineers, MMCalc works with a variety of number types, including real numbers, complex numbers, real-number intervals and gaussian numbers. Hundreds of units and physical constants are already built into MMCalc, and the user can add units as well. MMCalc offers unit calculations with automatic unit factoring and dimensional analysis. The program has a large stack for its numbers and works in a reverse Polish notation style that should be familiar to users of Hewlett Packard calculators. MMCalc parses algebraic expressions, allowing expressions to be typed in just as they are written. While providing all of the standard arithmetic, logarithmic and trigonometric functions, many other useful functions are built in, such as factoring, greatest common divisors, modulo, Bessel and gamma functions, and other number theory functions. Numbers can be input and viewed in binary, octal, decimal, hexadecimal, and with their corresponding ASCII characters. MMCalc for the Mac fully utilizes the Standard Apple Numerics Environment with its IEEE compliance and costs \$99 (US).

Support for Microsoft Windows, Electronic Handbooks (which serve as an online reference library for instant access to standard formulas and constants) and symbolic calculations for computer algebra are features of Mathcad 3.0, MathSoft's **technical calculations software package** (*Reader Service No. 117*). Mathcad handles complex problems that are beyond the scope of spreadsheets and unwieldy to program in FORTRAN or C. Examples include complex arithmetic, fast Fourier transforms, symbolic factoring of polynomials, Bessel functions, matrices, simultaneous linear and non-linear equation solving statistics and



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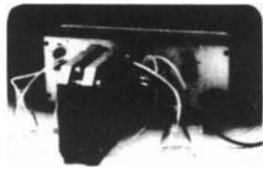
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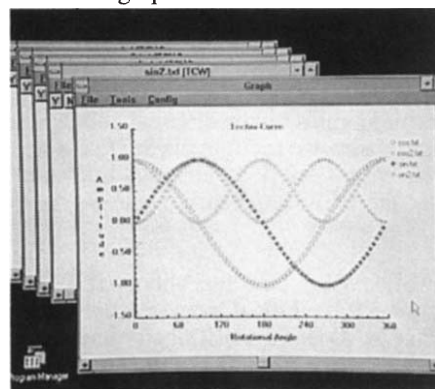
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other calculations. Other new features include improved equation editing, enhanced plotting, improved memory support, built-in units, and additional numerical functions. The 'live document interface' lets users work with Mathcad 3.0 and other MathSoft products in the same way they would use a scratchpad. Engineers, scientists and educators can combine equations using familiar mathematical notation with explanatory text and graphics in a 'live' document. Mathcad 3.0 costs \$495 (US).

Dealing with data

Techni-Curve is a new Windows-3.0 version of **technical graph plotting and curve-fitting software** from Aston Scientific (Reader Service No. 118). The software provides a complete range of technical graph formats such as x-y, bar chart and histograms, and allows large amounts of independent data to be loaded, manipulated and graphed. Data can be entered



Curve-fitting software from Aston Scientific.

manually or imported from spreadsheets, databases, or even directly from instruments. The £295 (UK) package provides complete control over all graph parameters such as axis limits, scaling, line thickness, labels, fonts, symbols and colours using the Windows point-and-click approach. Over 20 data sets can be superimposed on each graph and multiple graphs can be plotted on each page. In addition, Techni-Curve offers extensive regression and interpolation curve-fitting routines,

statistics and data transform capabilities, together with quantitative functions such as area measurement and the generation of calibration curves with automatic evaluation of unknowns.

SigmaPlot, Jandel's **scientific graphing software**, is now available in a Macintosh version (Reader Service No. 119). The Mac version includes a full



Jandel gets graphical.

range of scientific graphing features, including error bars, grids, axis breaks, regression lines, confidence intervals and quality-control lines. Data can be input directly from the keyboard, or from Excel, text and SigmaPlot (PC version) files, into a huge worksheet (16,000 columns by 32,000 rows). The software includes an extensive mathematical transform language and a powerful nonlinear curve fitter, Jandel says. The DM1,290 (Germany) SigmaPlot package also offers control over all independent graphic elements and a page-layout system that is similar to desktop publishing systems. Graphs can be sent to any output device supported by the user's Macintosh, or exported to PICT or EPS files.

If you ever look at a graph, chart or drawing and wish that you could easily **obtain the x, y data** from which it was drawn, Biosoft may be able to help. Now, users can scan most graphs, charts, drawings or lines with a scanner or video camera that outputs black and white TIFF or PCX files and UnGraph version 1.0 will supply its x, y coordinates with a level of precision specified by the user (Reader Service No. 120). This, Biosoft says, eliminates the need to use mechanical digitizing pads, which can be tedious, time-consuming and inaccurate. Designed for IBM PC/XT/AT/386/486/PS2 or compatible computers, the £199 (UK)/\$399 (US) UnGraph package has an automatic line follower to do the hard work (or you can use the mouse to follow a line manually). Results are in ASCII/DXF formats. □

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